Development of an *In Vitro* Diagnostic Technique for *Malassezia furfur*

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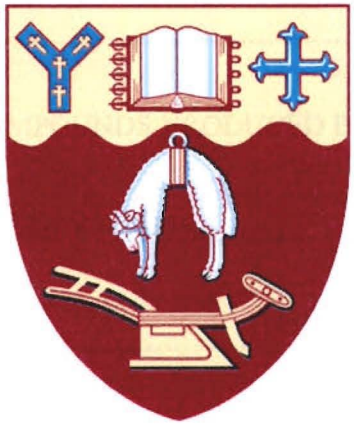

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ABSTRACT

Malassezia furfur (synonym *Pitryosporum ovale)* is a dimorphic, lipophilic yeast that is a commensal agent on human skin. *M. furfur* causes the skin conditions pityriasis versicolor, *Malassezia* folliculitits and seborrhoeic dermatitis. It also contaminate catheters supplying lipid emulsion which can led to pulmonary infections or septicaemia. Due to the increasing number of immunocompromised patients, the frequency and severity of *M. furfur* is escalating. Difficulty in culture is due to limited knowledge about the nutrient requirements of *M. furfur*, which leads to erroneous diagnosis and incorrect treatment.

The pathogenic nature of *M. furfur* led to investigations into the contagious nature of *M. furfur* infections. Some nutrient requirements of *M. furfur* were investigated using various commercially available media, in addition to other media developed from this work. The inhibitory effects of antifungal agents were investigated. The degradation of its cell wall was investigated using various cellulitic enzymes.

Results indicate the possible transferral of *M. furfur* infections from dead skin cells. *M. furfur* appears to be able to be cultured on a variety of commercially available media provided a lipid source is present. A modified bioMérieux ID32C Identification System could be useful for rapid identification of *M. furfur*. *M. furfur* also requires taurocholic acid for optimum growth and is inhibited by thiarubrine A. The results show that *M. furfur* is not difficult to culture, therefore, laboratories should be able to accurately identify the organism and thus assist clinicians in the diagnosis and treatment of conditions caused by this yeast.

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1. INTRODUCTION

Medical mycology is the study of medically important fungal infections in humans. The hair, skin and nails constitute a major area of recognisable fungal infection in humans. Fungal skin infections can be divided into Systemic and Superficial mycoses (Arnold *et al.*, 1990). Systemic mycoses are fungal infections, often of pulmonary (lung) origin, which usually give rise to limited lesions on the skin. Superficial mycotic skin infections are those that reach only a few millimetres into the skin tissue (Figure 1:4). Probably the most well known of these is athlete's foot (tinea pedis) caused by *Trichophyton* and *Microsporum* spp. Other superficial infections include tinea unguium (*Microsporum canis* and *Epidermophyton* species) which infects the fingernail; and tinea capitis and tinea barbae (*Epidermophyton floccosum*) which are infections of the scalp and beard respectively (Arnold *et al.*, 1990).

Yeast infections may also occur in the skin and surrounding areas, candidiasis (*Candida albicans)* being the most important. Other skin infections caused by *C. albicans* include perlèche and pseudonappy (diaper) rash (Arnold *et al.*, 1990) and onychomycosis (Faergemann, 1996; Richardson, 1997). Pityriasis versicolor (tinea versicolor), seborrhoeic dermatitis and *Malassezia* folliculitis are caused by *Malassezia furfur* (Arnold *et al.*, 1990; Faergemann *et al.*, 1996; Mayser *et al.*, 1995).

1.1 The Genus *Malassezia*

The genus *Malassezia* consists of seven species (Faergemann, 1997; Guého *et al.*, 1996). *M. furfur* is restricted to the human host, *M. sympodialis* is a cat and human pathogen (Bond *et al.*, 1996) and *M. pachydermatis*, the causative agent of canine otitis externa (Klein *et al.*, 1996) is also a human pathogen (Bond *et al.*, 1996). The remaining four species (*M. globosa, M. obtusa, M. restricta* and *M. slooffiae*) were recently isolated from infections previously believed to be caused by *M. furfur, M. sympodialis* or *M. pachydermatis* in origin (Guého *et al.*, 1996). Each new species was

identified by Guého *et al.* (1996) based on morphology, ultrastructure, physiology and molecular biology. All *Malassezia* species require a lipid source except *M. pachydermatis* (Ahern and Simmons, 1997; Faergemann, 1997).

Malassezia furfur (Baillon) is a dimorphic, lipophilic yeast and commensial on human skin (Hurley *et al.*, 1987; Klotz, 1989; Marcon and Powell, 1992; Riciputo *et al.*, 1996). As with many other microorganisms, when conditions are suitable *M. furfur* becomes pathogenic. The skin infections known to be caused by *M. furfur* are pityriasis versicolor (Faergemann, 1989), *Malassezia* folliculitis (Nyirjesy *et al.*, 1994; Sandin *et al.*, 1993) and seborrhoeic dermatitis (Ashbee *et al.*, 1993; Heng *et al.*, 1990; Ross *et al.*, 1994) and possibly atopic dermatitis (Faergemann, 1997) (Broberg *et al.*, 1992). *M. furfur* is also associated with intravenous catheter infections, particularly with catheters supplying lipid emulsions or meals (Barber *et al.*, 1993). The contaminated catheter can enable *M. furfur* to be spread internally by infected blood that can cause peritonitis and pulmonary infections (Ahern, 1997; Gidding *et al.*, 1989). Difficulty in culturing *M. furfur* leads to differential diagnosis and consequently, incorrect treatment.

1.2 Taxonomy of *Malassezia furfur*

The taxonomy of this dimorphic yeast has had a very confusing history indicating a great confusion in the nomenclature (Faergemann, 1989; Guého *et al.*, 1994). This was due, in some part, to the inability to obtain continuous cultures *in vitro* and since its nutrient requirements were not fully understood, all details of morphology were obtained from skin scrapings. Eichstedt, in 1846, identified a bran-like scaly skin condition he called pityriasis versicolor (tinea versicolor) (Sloof, 1970). Robin described the causative organism (note: organism(s) refers to a microbial species not a single cell) of psoriasis - identical to that to Eichstedt's description of pityriasis versicolor - as *Microsporon furfur* (Benham, 1947; Marcon and Powell, 1992) Benham (1947) mentions that the spelling of *Microsporon* was possibly incorrect and should have been *Microsporom.* In 1873, Rivolta reclassified *Microsporon furfur* as

Cryptococcus psoriasis and in 1884, Bizzozero renamed it *Saccharomyces sphaericus*. Sabouraud classified *Saccharomyces sphaericus* as a member of the blastomycetes in 1889 and renamed it *Pityrosporum malassezii* (Assaf and Weil, 1996; Cannon, 1986). However, in the same year, Baillon ignored Rivolta's and Bizzozero's classifications and transferred *Microsporon furfur* to a new genus *Malassezia* (Cannon, 1986) which he named in recognition of Malassez. Malassez distinguished the causative agent of pityriasis versicolor from those that cause tinea infections (Marcon and Powell, 1992). The plasma membrane of *M. furfur* is described as multilayered and ribbed (Keddie, 1966).

The first successful culture of the causative organism of pityriasis versicolor was in 1913 by Castellani and Chalmers which they named *Pityrosporum ovale* (Faergemann, 1989; Hurley *et al.*, 1987). Today there are two species which appear to be synonymous, *Pityrosporum ovale* and *Malassezia furfur*. In 1986, The International Commission on the Taxonomy of Fungi (ICTF) proposed a name change from *Pityrosporum ovale* to *Malassezia furfur*. Although this was not successful it was agreed that both *P. ovale* and *M. furfur* were synonymous (Cannon, 1986). It is thought that *M. furfur* is the invasive form producing hyphae (or pseudohyphae) (Marcon and Powell, 1992) and *P. ovale* the nonpathogenic form (yeast). Faergemann (1989) argues that the name *Malassezia furfur* is obtained from clinical symptoms of skin infections rather than conventional taxonomic techniques such as morphology and biochemistry.

In pityriasis versicolor it is thought that *M. furfur* changes morphology from yeast to mycelial form (Mayser and Grunder, 1995). The yeast and mycelial phase are often identified in the same lesion or culture so this is entirely possible (Figure 1:1) (Guého *et al.*, 1996). Nassaro-Porro *et al.* (1977) reported inducing hyphal growth from *M. furfur in vitro*. They used a combination of cholesterol, cholesterol stearate and glycerol monostearate at a ratio of 2:1.5:2 (Nassaro-Porro *et al.*, 1977). A simple test to induce hyphae in *C. albicans* is to inoculate the isolate into human sera (Ahern, 1997). To date no studies have been completed on the induction of *M. furfur* hyphae in human sera.

The yeast phase of *M. furfur* has been classified into three serovars, based on blastospore morphology and cell surface antigens. Serovars A and B have round blastospores whilst serovar C has oval blastospores. Previously, these serovars had been described as *P. orbiculare* (serovars A and B) and *P. ovale* (serovar C) (Cunningham *et al.*, 1992). This writer acknowledges that controversy still exists over the nomenclature but for the purpose of this work, the organism will be called *Malassezia furfur*.

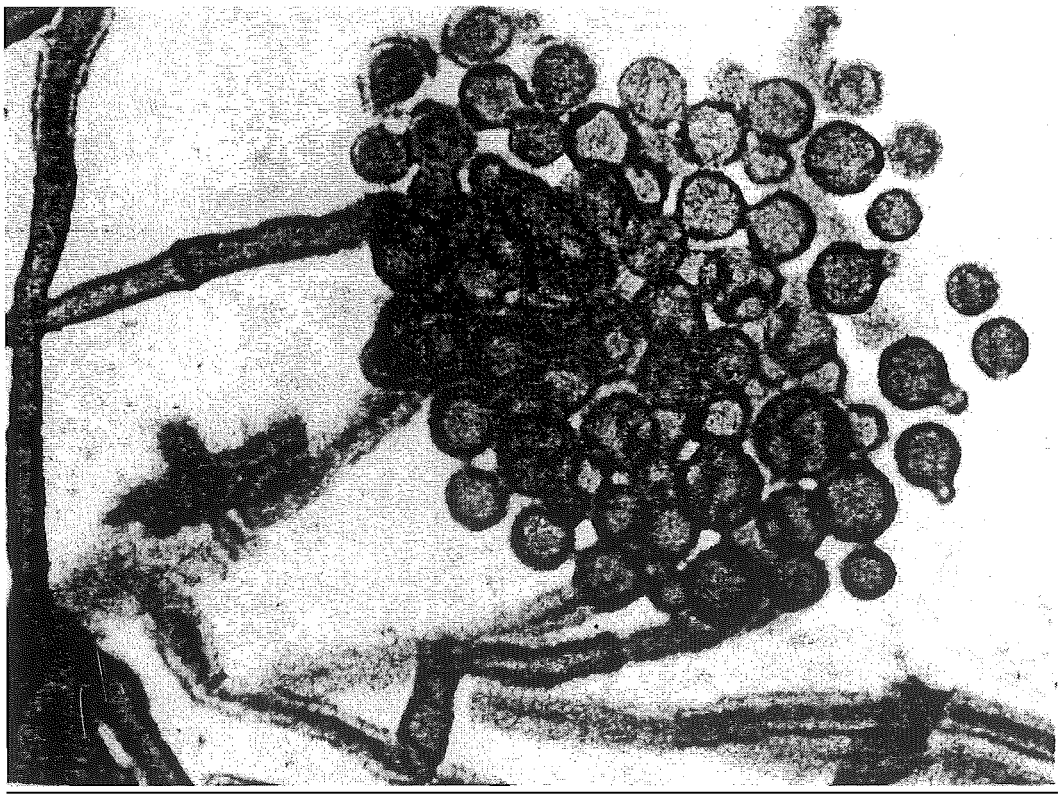


Figure 1:1 KOH mount of *M. furfur* infected skin scraping

Note the "spaghetti and meatballs" appearance (Arnold *et al.*, 1990)

1.3 Morphology

The yeast phase of *M. furfur* is globose to ellipsoidal to long-ellipsoidal about 4-5μm long (Figure 1:2) and reproduces through unipolar budding (Sloof, 1970). This unipolar budding leaves a pronounced scar or collar from where the buds appear (Ahern and Simmons, 1997). The synonym of *M. furfur, Pityrosporum ovale*, produces mycelia (or pseudomycelia) *in vivo*. *Malassezia furfur* colonies on Dixons Agar are dull, lobate, smooth in texture and cream coloured turning beige with age (Figure 1:3) (Marcon and Powell, 1992).

M. furfur is thought to be basidiomycetous in nature as indicated by the following characteristics: diazonium blue B staining, hydrolysis of urea, and absence of cell wall lysis by β 1-3 glucanase (Guého and Meyer, 1989). Investigations into the cell wall structure of *M. furfur* showed a thick cell wall with a regularly lobed plasma membrane (Keddie, 1966). The surface of the cell wall of *M. furfur* has a corrugated appearance (Ahern and Simmons, 1997). Direct examination of pityriasis versicolor with a Wood's lamp (UV) causes *M. furfur* to fluoresce yellow (Gealt *et al.*, 1989).

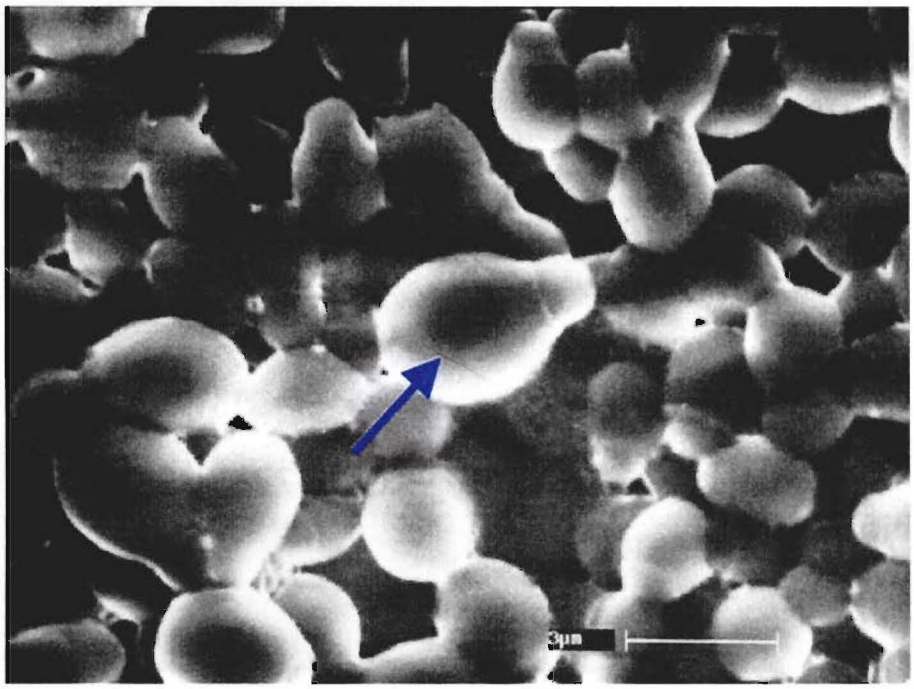


Figure 1:2 SEM of budding *Malassezia furfur*

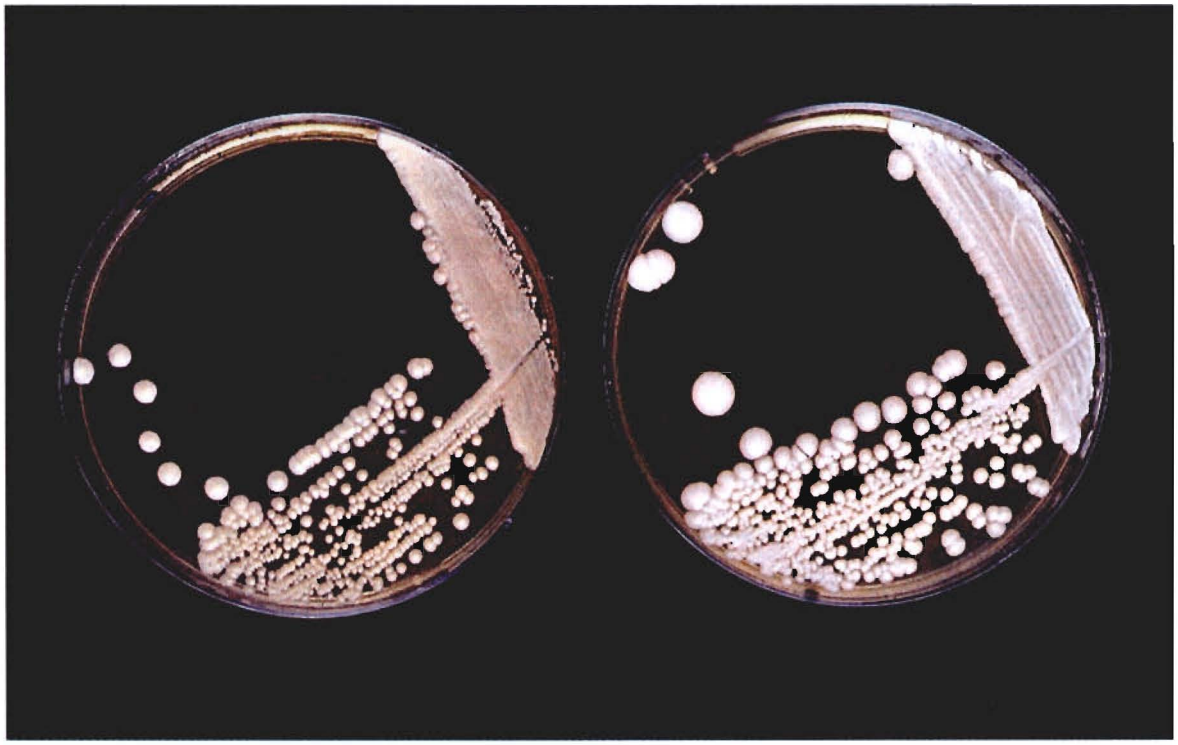


Figure 1:3 Morphology Comparison *Malassezia furfur* (left) and *Candida albicans* (right)

1.4 Pathogenicity

M. furfur is a common agent on human skin but under certain conditions becomes pathogenic. Environmental conditions such as increased temperature or humidity can increase the volume of skin secretion, which in turn may stimulate *M. furfur* growth. Application of oily substances, such as baby oil, onto skin may also contribute to a growth increase of *M. furfur* (Marcon and Powell, 1992). Increased sweating due to stress may also play a role in *M. furfur* infection as well as dietary problems, such as obesity (Noble, 1981). This yeast infects any age group regardless of race but adolescents have been found to have the highest rate of skin colonisation when the sebaceous glands become more active and the concentrations of lipid secretion increases (Marcon and Powell, 1992).

Skin infections caused by *M. furfur* are pityriasis versicolor, *Malassezia* folliculitits and seborrhoeic dermatitis. These conditions are not thought to be transferable or contagious (Marcon and Powel, 1992). **Pityriasis versicolor** is an infection of the stratum corneum layer (Figure 1:4) (Noble, 1981). The lesions are slightly scaly and papular or nummular and vary in colour from red to brown (Faergemann, 1997). A characteristic of pityriasis versicolor infections is a hypopigmentation or hyperpigmentation of the skin (Marcon and Powell, 1992). This change in skin colour is due to the inhibition of the enzyme tyrosinase, which is involved in melanin production. ***Malassezia* folliculitits** is characterised by follicular papules and pustules and chronic itching (Faergemann, 1997) and it is often confused with *Candida* folliculitits (Sandin *et al.*, 1993). **Seborrhoeic dermatitis** (dandruff is a mild form of seborrhoeic dermatitis) is described as greasy red to brown lesions (Faergemann, 1997; Faergemann *et al.*, 1996) and is frequent in AIDS patients (Ross *et al.*, 1994). **Atopic dermatitis** has long been associated with *C. albicans* however, it is now thought that *M. furfur* may play a role in this skin condition (Faergemann, 1997). *M. furfur* may also play a role in **acne vulgaris** lesions as it was found in 52%-68% of lesions biopsied from 71 cases by Lemming *et al.* (1989). *M. furfur* is classed as a superficial fungal infection because it does not appear to infect the deeper layers of the epidermis and

dermis (Marcon and Powell, 1992). It is also thought to infect hair follicles but not cause hair loss (Lopes *et al.*, 1994).

With the increase in immunosuppressive diseases such as AIDS and procedures that require suppression of the immune system, such as organ transplants, *M. furfur* infections are becoming more invasive. Deeper infections have also been reported through contaminated catheters particularly with intravenous lipid emulsions (Barber *et al.*, 1993). Initially, catheter-related sepsis was associated with neonates receiving lipid emulsions (Rapelanoro *et al.*, 1996). Now, it is known that *M. furfur* will infect any age group whether they are receiving catheter supplied lipid emulsions or not (Barber *et al.*, 1993). Patients with a lowered immune system are prone to *Malassezia* folliculitits (Teglia *et al.*, 1991). AIDS patients with a CD4 cell count lower than 100/μl or cancer patients with an absolute neutrophil count of lower than 100/μl are at greatest risk of contracting any fungal infection (Perfect and Schell, 1996).

1.5 Habitat

Malassezia furfur inhabits the stratum corneum or horny cell layer of human skin (Figure 1:4) (Noble, 1981) and the lumen of the piolesebaceous glands (Marcon and Powell, 1992). The distributions of colonies on skin are greater in high sebum (fatty acids and oil) containing sites such as face, chest and back (Leeming *et al.*, 1989). *M. furfur* is thought to obtain its nutrients from secretions from human eccrine glands. These glands are connected to the hair follicle (Figure 1:5) where *M. furfur* is known to inhabit (Lopes *et al.*, 1994). The secretions from the eccrine gland contains a variety of fatty acids, amino acids and simple sugars (Noble, 1981). The skin slightly acidic (pH 5.5) and this acidity acts as a protective device and the skin temperature is about 33.5 ° C (Noble 1981). The moisture content of the skin surface has two sources, through the eccrine glands and transepidermal water loss. The variety of lipids secreted onto the skin are in low amounts but this increases with the onset of puberty.

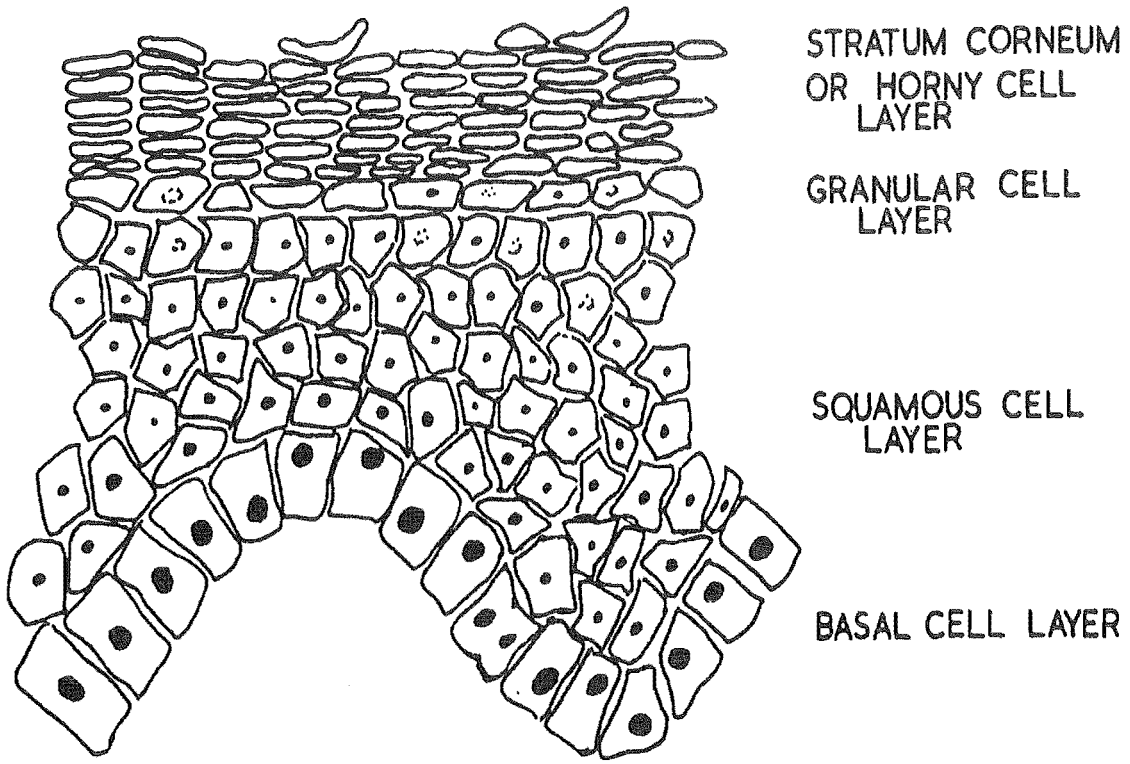


Figure 1:4 Diagrammatic representation of epidermal layer of human skin (Noble 1981)

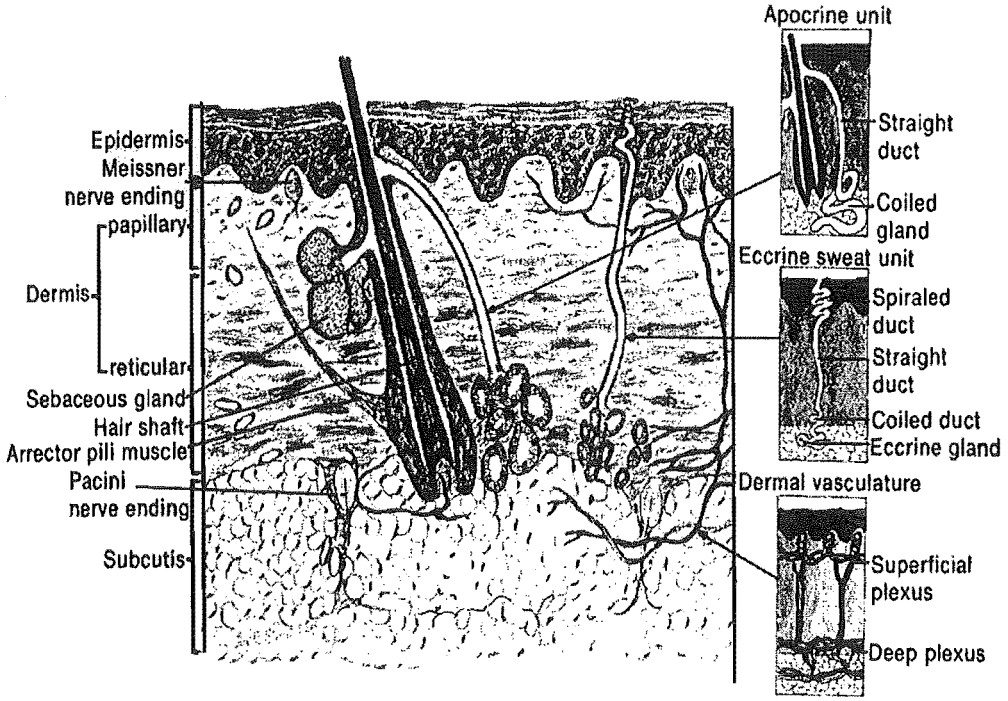


Figure 1:5 Diagrammatic cross section of human skin (Arnold *et al.*, 1990)

1.6 Growth of *Malassezia furfur in vitro*

All members of the genus *Malassezia* require lipid supplementation except *M. pachydermatis*. Guého *et al.* (1996) investigated each species' ability to utilise different lipid requirements. *M. furfur* and *M. sympodialis* are the only two species being dependent on the presence of a fatty acid source (Guého *et al.*, 1996; Marcon and Powell, 1992). The two species can be distinguished by their assimilation of polyoxyethylene sorbitan monolaurate (Tween 20®) because *M. furfur* assimilates Tween 20® but *M. sympodialis* does not (Guého *et al.*, 1996).

M. furfur requires medium to long chain fatty acids for growth (C12-C24) as it is unable to synthesise them *de novo* (Faergemann, 1989). Lipids isolated from skin secretions are mainly C18 and include oleic acid, palmitic acid, lauric acid, myristic acid and stearic acid (Noble, 1981). The concentrations on skin are considerably lower than those supplied in media. Oleic acid, for example, is found in skin secretions at a concentration of 0.002 g/l but in culture media, it is usually provided at concentrations of 1.0 g/l (Van-Gerven and Odds, 1995). In growth media lipid sources have been supplied in the following forms

Table 1:1 Some *In vitro* Lipid Sources for *Malassezia furfur*

Some Lipid Sources	Reference
Butter and lecithin	Benham (1947)
Glycerol	Van-Gerven and Odds (1995)
Polyoxyethylene sorbitan monolaurate (Tween 20®)	Guého *et al.* (1996)
Polyoxyethylene sorbitan monopalmate (Tween 40®)	Guého *et al.* (1996)
Polyoxyethylene sorbitan monooleate (Tween 80®)	Guého *et al.* (1996)
Olive oil	Nyirjesy et al. (1994)
PEG-7 glycerol monoalcarioate (Cetiol HE™)	Mayser *et al.* (1997)
PEG-glyceryl sterate (Tagat S2™)	Mayser *et al.* (1997)
PEG-3,5 castor oil (Cremophor EL™)	Mayser *et al.* (1997)
PEG-9 sterate (Cremophor S9)	Mayser *et al.* (1997)

Lipids are required to maintain membrane function and structure, for production of hormones and toxins, and storage of energy in the form of triglycerides (Gealt *et al.*, 1989).

Also required for optimal growth *in vitro* are bile salts and malt extract. The major component of bile salts is taurocholic acid, which aids in fatty acid decomposition (Benmoussa *et al.*, 1993; Momose and Tsubaki, 1997). Malt extract contains a variety of simple sugars and nitrogen plus concentrations of calcium (140 ppm) and magnesium (703 ppm). The nutrient requirements of *M. furfur* still needs to be addressed further in the form of the ability to assimilate carbohydrates and use of other possible nitrogen sources such as amides.

As previously stated, *M. furfur* requires an exogenous lipid source and because of this expresses various lipid degrading enzymes to utilise these fatty acids (Mayser *et al.*, 1996) (Mayser *et al.*, 1995). The breakdown of polyoxyethylene sorbitan esters is accomplished by hydrolases. However, other fatty acids such as glycerolesters are degraded by lipases (Mayser *et al.*, 1996). Extracellular enzymes reported to be produced by *M. furfur* are amylase, RNase, polygalacturonase, pectin lyase, proteases and urease (Petruccioli and Gallo-Federici, 1992) lipase and esterase (Plotkin *et al.*, 1995). *M. furfur* has phospholipid degradation activity as shown by Riciputo *et al.* (1996) and they demonstrated that *M. furfur* will grow on a medium containing egg yolk as the only lipid. This phospholipase (unspecified) is inhibited by magnesium sulphate but the addition of Tween 60 ® allowed for successful growth of *M. furfur* (Riciputo *et al.*, 1996).

1.7 Identification

Erroneous diagnosis of *M. furfur* is frequent and skin symptoms are often not sufficient for a correct diagnosis. Pityriasis versicolor has been incorrectly identified as seborrhoeic dermatitis, pityriasis rosea, pityriasis alba, leprosy, syphilis and vitiligo (Arnold *et al.*, 1990). Crude identification methods for *M. furfur* include; skin scrapings stained with Parker Ink-KOH (Sei *et al.*, 1994) and placing contact agar plates onto infected areas which are then cultured (Faergemann, 1987). There is conflicting evidence in the number colony forming units in relation to infection. Faergemann (1987) suggests that the numbers of yeast cells found in infections are the same as in normal skin tissue. Therefore, methods such as contact plates are not useful. However, others disagree and state that there is a significant rise in the number of colony forming units in skin infections caused by *M. furfur* (Bergbrant *et al.*, 1992). Antibodies for *M. furfur* have been identified (IgE and IgG) (Faergemann, 1989) and are now incorporated into skin colonisation studies (Broberg *et al.*, 1992)

Identification of suspected *M. furfur* infections from blood samples are more difficult as attempts at obtaining cultures are often unsuccessful (Marcon and Powell, 1992). Lyon *et al,* (1995) compared the Bact/Alert and Isolator Blood Culture Systems to assess whether either technique was effective in identifying fungal cultures from blood samples (including *M. furfur*). The Isolator Blood Culture System proved quite effective in identifying *M. furfur* yet the Bact/Alert was not as effective (Lyon *et al.*, 1995). The polymerase chain reaction (PCR) can be used for typing *M. furfur* strains (Makimura *et al.*, 1994; Van-Belkum *et al.*, 1994). The latest success is the identification of the first allergenic protein of *M. furfur* (Schmidt *et al.*, 1997). This protein causes an increase in the anitbodies IgE and IgG in patients with *M. furfur* infecitons. This information could lead to a molecular based identification technique for *M. furfur*

1.8 Control

Many antifungal agents have been investigated or prescribed for fungal infections of the skin. Coal Tar Gel (Wright *et al.*, 1993) and selenium sulphide (Marcon and Powell, 1992) are used in soaps and shampoos and are an effective prophylactic treatment. Lamisil ™ cream, which has the antifungal agent terbinafine, has also been reported to be effective (Evans *et al.*, 1994). Another possible prophylactic treatment for *M. furfur* is the topical use of the essential oil from *Melaleuca alternifolia* (tea tree oil) (Nenoff *et al.*, 1996). This oil has been found to inhibit *M. furfur in vitro* at concentrations of 0.12 % (Hammer *et al* 1997). This concentration is considerably less than those supplied in conventional oil soaps and lotions. The active compounds in tea tree oil are thought to be terpinen-4-ol, α-pertineol and α-pinene (Raman *et al.*, 1995). Another alternative could be single oral dose of fluconazole for the treatment of *M. furfur* skin infections (Faergemann, 1992).

Frequent recurrence of infection is a common problem with *M. furfur.* Superficial infections caused by infected catheters can often be eliminated by the removal of the catheter (Barber *et al.*, 1993). Deeper infections whether they originated from contaminated catheters or not, are more difficult to treat. There are many antifungal agents available aid in the control of more invasive forms of *M. furfur.* These include flutrimazole, ketoconazole, miconazole and sertaconazole (Van-Gerven and Odds, 1995), amphotericin B and natamycin (Adams, 1987). Many of these antifungal agents have severe side effects that in turn limit the concentration and duration of use. Other antifungal agents such as thiarubrine A have been shown to be effective against *C. albicans* and *Saccharomyces cerevisiae* (Constable and Towers, 1989) but to date studies on this antifungal agent have not included *M. furfur.*

Limited information is available about the cell wall composition of *M. furfur.* Most knowledge of yeast cell wall composition has been obtained from *Saccharomyces cerevisiae* (Current *et al.*, 1995; Georgopapadakou and Tkacz, 1995; Wheals, 1987). The fungal cell wall consists primarily of carbohydrates such as glucan polymers (1-3

and 1-6 β linked D-glucose), chitin and α-mannan (Current *et al.*, 1995; Munro and Gow, 1995).

As noted previously, *M. furfur* is resistant to cell wall lysis by β 1-3 glucanase, therefore, other cell wall degrading enzymes effective against *M. furfur* need to be investigated. The degradation of the fungal cell wall is an area of current interest with respect to antifungal agents.

1.9 Antimicrobial Compounds Produced by *Malassezia furfur*

M. furfur is thought to produce azelaic acid (Hay, 1993; Nassaro-Porro and Passi, 1978) which gives the characteristic change of pigmentation in pityriasis versicolor by inhibiting hydroxylation of l-tyrosine to l-dopamine (Nassaro-Porro *et al.*, 1977; Passi *et al.*, 1991; Wood, 1990). Azelaic acid is a naliphatic, dicarboxylic acid which has been shown to be effective in treating some skin diseases such as acne and benign hyperpigmentation. It has been incorporated into cosmetic surgery procedures (Passi *et al.*, 1989; Lowe, 1996). The antibacterial activity of this acid has been investigated by with varying minimum inhibitory concentration (MIC) from 0.03 M/l to 0.25 M/l (Leeming *et al.*, 1986). However, it has been reported that azelaic acid inhibits *P. ovale* (the hyphal form of *M. furfur*) at levels of $3x10^{-2}$ M/l to 5.3 10^{-1} M/l (Brasch and Christophers, 1993). To date no other antimicrobial or antitumor compounds have been identified from *M. furfur*.

Malassezia furfur has been reported to be difficult to grow and therefore to identify and cure. The conflict in nomenclature combined with the lack of understanding about it's nutrient requirements. Differential diagnosis and the chronic nature of infections leads to a less than satisfactory situation for the treatment of these conditions. The aim of the present work is to develop a rapid, workable, diagnostic technique and to assess the efficiency of a number of antifungal agents.

2. Materials and Methods

2.1 Culture

For the purpose of this work the fungus *Malassezia furfur* NZRM-3493 was obtained in freeze-dried form from the Communicable Disease Centre (CDC), Wellington, New Zealand. This culture was originally from a patient with an infected fingernail isolated at Wellington Hospital in 1995. Samples of skin infected with *M. furfur* were obtained from Canterbury Health Laboratories, Christchurch, New Zealand.

2.1.1 Culturing of Freeze-Dried Isolate

Malassezia furfur was rehydrated following the instructions supplied by CDC. All cultures, unless specified, were inoculated onto Dixon's Agar (See Appendix I). The following three methods were assessed to in order to identify the most effective culturing technique. The number of colony forming units per plate determined this.

- Approximately 100 µg of freeze-dried *M. furfur* culture was inoculated onto Dixon's Medium and incubated at 37°C for 48 hours.

- Approximately 100 µg of dried *M. furfur* culture was inoculated into 10 ml of Dixon's broth (See Appendix I) and incubated for 24 hours at 37°C. Dixon's Medium was inoculated with 100 µl of Dixon's Broth culture containing *M. furfur*, aseptically spread and allowed to air dry. Plates were incubated at 37°C for 48 hours.

- The remainder of the freeze-dried *M. furfur* was emulsified in 10 ml of Dixon's Broth and incubated for 24 hours at 37°C. Dixon's Medium was inoculated with

600 µl of the Dixon's Broth culture, aseptically spread and allowed to dry. Plates were incubated at 37°C for 48 hours.

The remaining inoculum was incorporated into a 50% glycerol solution, placed in cryogenic tubes and frozen at -80 ° C for future use.

2.1.2 Culturing of Skin Isolate

It is thought that *M. furfur* infections are not transferable. these is the possibility that *M. furfur* infections could be transferred using dead skin as a vector. Therefore, skin scrapings that had previously been identified as having a *M. furfur* infection (courtesy of Ros Podmore, Canterbury Health Laboratories) were assessed for viability. These skin scrapings were very dehydrated so two protocols were investigated:

- Small pieces of dehydrated skin were placed directly onto Dixon's Agar amended with cycloheximide, chloramphenicol and gentamicin (DMCCG) (See Appendix I). Plates were incubated at 37°C for 48 hours.

- Five small pieces of dehydrated skin were rehydrated in 10 ml of sterile water for one hour and each piece was streaked across DMCCG using sterile forceps. The rehydrated skin was left on the medium and incubated at 37° C for 48 hours.

2.2 Identification Methods for *Malassezia furfur*

Due to the basidiomycetous nature of *M. furfur* (Guého and Meyer, 1989), basic biochemistry tests were completed to confirm the identity of the CDC culture. *M. furfur* is thought to hydrolyse urea (Guého and Meyer, 1989) and growth on Sabouraud Dextrose Agar (SDA) amended with olive oil is a common medium used for isolation of *M. furfur* cultures (Ros Podmore, pers com, Canterbury Health Laboratories, 1996) (Atlas, 1993; Nyirjesy *et al.*, 1994). A common test for urea hydrolysis is to culture the microbial species on Christensen's Urea Agar (See Appendix I) which contains a pH indicator, phenol red. A positive reaction due to a pH change from neutral (pH 7) to alkaline (pH 8.5) results in a colour change from orange to pink.

- Christensen's Urea Agar was inoculated with 600 µl suspension of *M. furfur* in Dixon's broth, aseptically spread, left to dry and incubated at 37°C for 48 hours.

- SDA with olive oil (See Appendix I) is a common medium used for growth of *M. furfur* (Atlas, 1993). SDA and SDA amended with olive oil were inoculated with *M. furfur* using the same protocol as described above. *M. furfur* should not be able to grow on SDA due to the lack of a lipid supplement (olive oil)

2.3 Growth Media

Part of this work was to develop a rapid diagnostic technique for *M. furfur*. Therefore, in order to quickly appraise various media, a simple method was employed that enabled the medium to sustain more that one type of organism. Initially a sterile loop was placed in the desired culture and streak inoculated across the medium once (Figure 2:1). If this preliminary method gave a positive result for example the desired colour change, a known concentration of yeast suspension was inoculated onto appropriate medium (see below). The suspension involved the addition of a single loop of solid yeast culture inoculated into 100 ml of Dixon's Broth. This was then incubated at 37°C for 24 hours in a New Brunswick Scientific Shaking Incubator at 100 rpm.

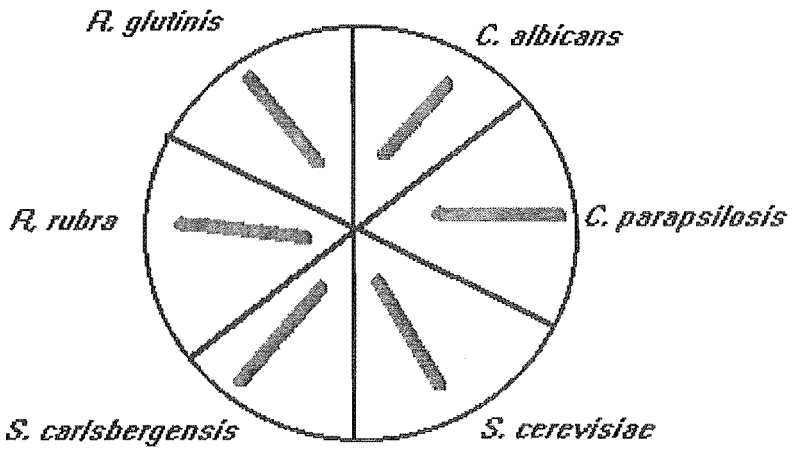


Figure 2:1 Representation of Streak Inoculation Test

2.3.1 Media Incorporating Indicators

As previously reported (Guého and Meyer, 1989) *M. furfur* hydrolyses urea. Some commercially available urea based media were inoculated with *M. furfur* to ascertain the ability for *M. furfur* to grow on different medium. The preliminary tests (see previous page) were performed and cultures were incubated at 37°C for 48 hours.

2.3.1.1 Utilisation of Urea

Preliminary tests were carried out on Phenol Red Agar, Urea Agar and Dixon's Medium amended with Christensen's Urea Salts.

- These growth media were streak inoculated with *M. furfur* or the comparison (control) yeasts; *Candida albicans* CANU-C57, *C. parapsilosis* MCC499 (CDC, Atlanta, USA, 1977), *Rhodotorula glutinis* CANU-C117, *R. rubra* CANU- C204, *Saccharomyces carlsbergensis* CANU- C95, and *S. cerevisiae* 329-6C and incubated at 37°C for 48 hours.

2.3.1.2 Utilisation of Nitrogen Sources

The utilisation of a variety of nitrogen sources by fungi can be used as a taxonomic aid (Sloof, 1971). The number of reported nitrogen sources known to be used by *M. furfur* are few but often the concentrations of the nitrogen sources assessed were not disclosed (Benham, 1947). Various nitrogen sources were assessed using the preliminary test given above.

- Solutions of creatine, ammonium acetate and aspartic acid were made up at concentrations of 1%, 3% and 5% in 95% ethanol (Benham, 1947) and were incorporated into a Bromocresol Purple Agar (See Appendix 1). The amended

Bromocresol Purple Agar was streak inoculated with *M. furfur* or comparison yeasts and incubated at 37°C for 48 hours.

2.3.1.3 Utilisation of Amides

Amides can be utilised by yeasts as a nitrogen source. However, Mira-Gutierrez *et al.* (1994) did not assess amide hydrolysis by *M. furfur*, therefore further investigations into amide utilisation were included in this study.

- The following amides were added to 600 ml of Christensen's Urea Agar base at concentrations of 0.25%: acetamide, allylurea, benzamide, 2-chloroacetamide, formamide, isonicotinamide, malonamide, $_{L}$-phenylalaninamide, propionamide, salicilamide, thioacetamide, thiourea and urea (at 2%). This modified Christensen's Urea Agar was streak inoculated with *M. furfur* or comparison yeasts and incubated at 37°C for 48 hours.

2.3.2 Media Incorporating Lipids

M. furfur requires fatty acids for growth because of its inability to synthesise them *de novo* (Faergemann, 1989). The ability of *M. furfur* to utilise a variety of lipids, specifically, fatty acids (Tweens®) and other novel lipid sources such as egg yolk and milk was assessed.

2.3.2.1 Utilisation of Some Polyoxyethylene Sorbitan Esters (Tween ®)

- Tween 20®, Tween 40® and Tween 80® were added to Dixon's Medium at concentrations of 1%, 5%, 10%, 15% and 20% v/v. At concentrations of 15% and 20% the medium failed to solidify. To remedy this, the agar concentration was increased to 3%. The amended Dixon's Medium was spread inoculated with 600 µl

suspension of Dixon's Broth cultures of *M. furfur* or comparison organisms, allowed to air dry and incubated at 37°C for 48 hours.

2.3.2.2 Utilisation of Cholesterol, Egg Yolk and Milk

To extend the investigations into the lipid requirements of *M. furfur* some common lipid sources were assessed. In addition to media similar to those used by Nassaro-porro *et al.* (1977) and Mayser *et al.* (1996), a milk based agar was assessed.

- Bromocresol Purple Milk Agar, Cholesterol Agar and Egg Yolk Agar (See Appendix I) were streak inoculated with *M. furfur* or comparison organisms in Dixon's Broth and incubated at 37°C for 48 hours.

2.3.3 Media Incorporating Other Nutrients

2.3.3.1 Growth on Sweat Medium

The natural habitat of *M. furfur* is the skin and Noble (1981) discusses the sweat secretions of human skin, citing a sweat medium developed by Murphy (1975) which was based on the components of sweat. Attempts were made to assess this "Sweat Medium" for culturing skin organisms.

- Sweat Medium (See Appendix I) was streak inoculated with *M. furfur* plus comparison organisms, allowed to air dry and incubated at 37 ° C for 48 hours.

2.3.3.2 Utilisation of Taurocholic Acid

Oxgall ™ (Oxoid ™) or Bile (Sigma ™) is a component of Dixon's Medium of which a major component is taurocholic acid. This acid is involved in the degradation of fatty acids in mammals (Benmoussa *et al.*, 1993; Momose and Tsubaki, 1997). Investigations were carried out to assess the extent to which *M. furfur* requires this acid.

- Dixon's Medium was modified for this experiment with the omission of the desiccated bile. This modified Dixon's Medium was incorporated with taurocholic acid (instead of Oxgall™ or Bile) at concentrations of 0.5% 1.0%, 1.5%, 2.0% and 2.5%. Controls consisted of standard Dixon's Medium and Dixon's Medium without Oxgall or Bile. These plates were streak inoculated with *M. furfur* and comparison organisms as previously described.

2.3.3.3 Assessment of ID 32C Substrate Assimilation System

Nutrient requirements of *M. furfur* were further investigated using the bioMérieux Identification System for Yeasts. This system assesses carbohydrate assimilation.

- *M. furfur* was inoculated into the ID32C Identifications System® using the standard protocol (See Appendix II). As *M. furfur* requires a lipid source, 10% Tween 80® and 2.5% glycerol were incorporated into the suspension medium in the ID32C system. In further investigations, the suspension medium was replaced with a sterile solution of 25% Tween 80® and 25% glycerol and the standard protocol continued.

2.3.3.4 Gelatine Hydrolysis

The work by Petruccioli and Gallo-Federici (1992) assessed the ability of various yeasts to hydrolyse gelatine. In this work, the ability of *M. furfur* to hydrolyse gelatine was assessed by using a different protocol to that specified by Petruccioli and Gallo-Federici (1992).

- One millilitre aliquots of Nutrient Broth containing 12 % gelatine (See Appendix I) were distributed into 5 ml Bijoux bottles and autoclaved at 121°C for 20 minutes then left for 24 hours to set. Bottles were stab inoculated with *M. furfur* or comparison organisms and then incubated at 37°C for 24 hours. The inoculated Bijoux bottles were then incubated at 4°C for 20 minutes. Those media, which stayed liquid after that time, gave a positive result of being able to hydrolyse gelatine. Samples, which solidified, were placed in a 30°C incubator on a slant for 20 minutes. Those, which showed signs of liquefying, indicated mild gelatine hydrolase activity.

2.4 Induction of the Hyphal Phase of *Malassezia furfur*

One of the main taxonomic anomalies concerning *M. furfur* is the inability to obtain hyphal growth *in vitro* (Marcon and Powel, 1992). Nassaro-Porro *et at.* (1977) induced hyphal production in *M. furfur* by using a modified cholesterol agar. *C. albicans* can be quickly identified by the production of pseudohyphae when inoculated into human sera (Ahern, 1997). The phase of *M. furfur* is only ever been found *in situ*, therefore, investigations were conducted to see if *M. furfur* is stimulated into hyphal phase by human sera.

- Cholesterol Agar (See Appendix I) was inoculated with 600 µl of Dixon's Broth culture of *M. furfur* and incubated at 37°C for 48 hours. No controls were performed because the comparison organisms had already been assessed in section 2.3.2.2.

- A flame-sterilised platinum loop was placed in a 24 hour solid culture of *M. furfur* and *C. albicans* respectively then inoculated into separate sterile Bijoux bottles containing 2 ml of human sera (obtained from the Christchurch Medical School) and incubated at 37°C for 24 hours.

2.5 Response to Inhibitory Agents

Van Gerven and Odds' (1995) thoroughly investigated the response of *M. furfur* to imidazole antifungal agents which were not considered in the present study. *M. furfur* is thought to be resistant to some commonly used antifungal agents (Adams, 1987) so further studies with some were carried out. It is well documented that *M. furfur* is resistant to cell wall lysis by β 1-3 glucanase. In an attempt to find an effective inhibitor of *M. furfur* other fungal cell wall degrading enzymes were assessed.

2.5.1 Antifungal Agents

Some common antifungal agents were tested against *M. furfur*. It has been reported that amphotericin B and Nystatin are not effective against *M. furfur* (Adams, 1987). Amphotericin B has very strong inhibitory effects on filamentous fungi (Brajtburg *et al*, 1990). Nystatin is thought to be specific against *C. albicans* (Kerridge and Whelan, 1984) and if incorporated into a specific medum (like SDA with antibiotics) would inhibit *C. albicans* growth, thus moving closer to obtaining a selective media for *M. furfur*. These antifungal agents as well as other commercially available fungicides and one novel antifungal agent were assessed for inhibitory activity by using either an agar dilution series or assay or both.

2.5.1.1 Amphotericin B Sensitivity

- An agar dilution series was carried out using Dixon's Medium. Dixon's Medium was amended with amphotericin B solution (See Appendix II) at concentrations of 5.1 mg/ml, 2.5 mg/ml or 1 mg/ml and streaked inoculated with *M. furfur or* comparison organisms and incubated at 37 ° C for 48 hours.
- To test the activity of amphotericin B, the above medium was plug inoculated with *Aspergillus fumigatus* CANU-F1, *A. niger* CANU-F27, *Fusarium oxysporum* CANU-F14 and incubated as previously described.

- Further work led to an amphotericin B assay. Dixon's Medium was inoculated with 600 µl suspension of Dixon's Broth cultures of *M. furfur* plus comparison organisms and allowed to dry. Sterile 6 mm bioassay disks were inoculated with 14 µl of amphotericin B solution at concentrations of 10^{-4} M, 10^{-5} M and 10^{-6} M. The inoculated disks were placed on the air-dried plates and incubated at 37°C for 48 hours. A positive control for this method was difficult to obtain because it is rare to find fungi that are resistant to Ampnotericin B

2.5.1.2 Nystatin Sensitivity

- Nystatin solution (See Appendix II) was incorporated into Dixon's Medium using the agar dilution series as for amphotericin B. Aliquots of Nystatin solution at concentrations of 5 mg/ml, 2.5 mg/ml and 1 mg/ml were added to Dixon's Medium. This amended medium was streak inoculated with cultures with *M. furfur* and comparison organisms and incubated at 37°C for 48 hours.

- As described previously, a Nystatin Assay was conducted using the equivalent concentrations mentioned above.

2.5.1.3 Thiarubrine A Sensitivity

The antifungal activity of Thiarubrine A *in vitro* was evaluated. The chemical structure of thiarubrine A is altered when exposed to light, therefore, the following method was performed in minimal light.

- Dixon's Medium plates were inoculated with 600 µl Dixon's Broth culture of *M. furfur* or comparison yeasts and allowed to air-dry. Sterile bioassay disks were inoculated with thiarubrine A at concentrations of pure concentrate (10^{0}), 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} with a control of acetonitrile (100 %) and placed on the air dried plates and incubated at 37 ° C for 48 hours.

- Thiarubrine A is more effective as an antifungal agent if exposed to light, when it is chemically transformed to thiophene (Constable and Towers, 1989). The above protocol was repeated with thiarubrine A exposed to white light for the duration of the experimental work (approximately 30 minutes).

2.5.1.4 Agricultural Fungicide Sensitivity

Agricultural fungicides (Table 2:1) were incorporated into 300 ml aliquot's of Dixon's Agar at concentrations recommended by the manufacturer. 600 µl of Dixon's Broth with *M. furfur* or test organisms was inoculated onto these plates and incubated at 37 ° C for 48 hours.

Table 2:1 Agricultural Fungicides

Fungicide	Concentration	Chemical Group
Alleate™	2.5 g/l	Fosethyl-aluminium
Bravo™	3.0 ml/l	Chlorothalonil (nitrate)
Captan™	2.0 g/l	Sulfenimide
Copper oxychloride	5.0 g/l	Copper – metal
Manzeb™	2.0 g/l	Dithiocarbamate
Saprol™	1.9 g/l	Triforine (piperazine)
Zineb™	2.0 g/l	Dithiocarbamate
Greenguard™	5.0 ml/l	Chlorothalonil and Thiophanate-methyl

2.5.2 Effect of Fungal Cell Wall Degrading Enzymes

Fungi are eucaryotic organisms and therefore targets for antifungal activity are limited but the cell wall does offer a potential target. Research on inhibition of fungal cell wall synthesis and cell wall degradation is considerable (Current *et al.*, 1995; Georgopapadakou and Tkacz, 1995; Munro and Gow, 1995). Guého and Meyer (1989) discussed some taxonomic characteristics of *M. furfur* and one of these was the resistance of *M. furfur* to cell wall lysis by β 1-3 glucanase. The action of other cell wall degrading enzymes will be assessed.

- Aliquot's (250 µl) of Dixon's Broth cultures of *M. furfur* or comparison organisms were inoculated into sterile microtubes and spun at 13 400 x g for 2 minutes. The supernatant was removed and the pellet suspended in 20 µl of 1M Phosphate Sorbitol Buffer Solution (PSBS) (See Appendix II). Various cell degrading enzymes (Table 2:2) at concentration of 10 mM/ml were added and the cultures were incubated at 37°C overnight. The cultures were then inoculated (approx 50µl) onto Dixon's Medium, aseptically spread, left to air dry and incubated at 37 ° C for 48 hours.

- Dixon's Medium plates were inoculated with 600 µl of Dixon's Broth cultures of *M. furfur* or control organisms. The broth was aseptically spread and left to air dry. An assay was carried out using β 1-3 glucanase at concentration of 750 U/ml, 500 U/ml, 250 U/ml, 100 U/ml, and 75 U/ml in 1 M Phosphate Buffer Solution (PBS) (See Appendix II). 14 µl aliquot's of β 1-3 glucanase were inoculated onto sterile disks with a control disk of sterile distilled water. The disks were placed on the inoculated Dixon's Agar plates and incubated at 37°C for 48 hours.

Table 2:2 Fungal Cell Wall Degrading Enzymes

Enzyme	Source	Activity
Driselase (Sigma)	Basidiomycetes	Laminarinase, xylanase and cellulase
β-Glucosidase (Sigma)	Almonds	β glucosidase
Lyticase (Sigma)	*Arthrobacter luteus*	β 1-3 glucanse
Lysozyme (Sigma)	Egg white	protease activity
α-Mannosidase (Boehringer Mannheim)	Almonds	contains mannosidase
Novozyme (Lysing enzyme L 2265, Sigma)	*Trichoderma harzianum*	cellulase, protease and chitinase activities

2.6 Antimicrobial Agents Produced by *Malassezia furfur*

Bacteria and fungi which live in the same habitat often compete for nutrients and space (Noble, 1981). This competition is sometimes maintained by the production of antimicrobial agents. *M. furfur* is believed to produce azelaic acid, and this acid has been incorporated into antibacterial creams for acne (Brasch and Christophers, 1993; Graupe *et al.*, 1996). An antimicrobial assay was conducted, as well as an ethyl acetate extraction of possible antifungal compounds from *M. furfur* was investigated.

2.6.1 Azelaic Acid Assay

Investigations were conducted to assess whether or not *M. furfur* produced any antimicrobial agents with activity against skin associated microorganisms. Dixon's Agar was inoculated with 600 µl suspension of Dixon's Broth culture containing *Streptococcus epidermidis* CANU-B121, *Staphylococcus aureus* ATCC 25923 (NZCDC #917) or *Microsporon canis* CANU-C203 and left to air-dry. Sterile 6 mm bioasssay disks were inoculated with 14 µl of either 20% azelaic acid solution (See Appendix II) or 14 µl of *M. furfur* in broth. Plates were incubated at 37°C for 72 hours.

Plates of Dixon's Medium were inoculated with single streaks of *S. epidermis, Staph. aureus* and *M. canis* cultures with *M. furfur* was streaked parallel to them (Figure 2:2). Sterile bioassay disks were inoculated with 14 µl 20% azelaic acid solution and placed at the other end of the streaks of *S. epidermidis, Staph. aureus* and *M. canis*. The plates were incubated at 37 ° C for 48 hours.

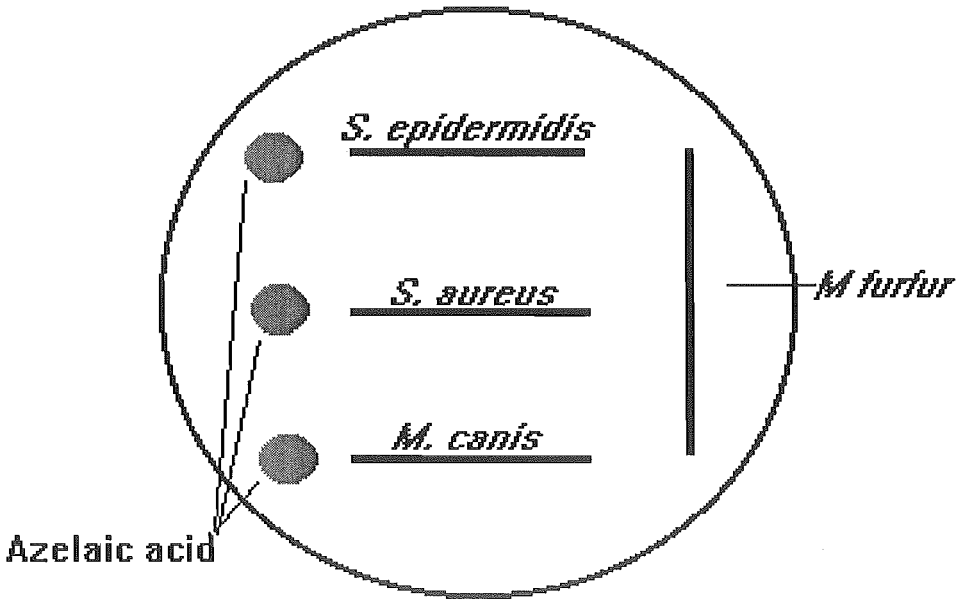


Figure 2:2 Antimicrobial Assay

2.6.2 Ethyl Acetate Extraction of Fungal Metabolites from Malassezia furfur

M. furfur was examined for any antimicrobial or antitumor activity.

1. A 24 hour Dixon's broth culture of *M. furfur* was homogenised in an Ultrataurex™ Blender for 1 minute, then filtered through celite and 22 μm filter to remove any cell debris. The celite was washed a further two times in ethyl acetate to ensure all the extract was removed.
2. The filtered broth was extracted with three washed of 30 ml 100 % ethyl acetate. Each time the ethyl acetate was removed and placed in a separate flask.
3. Once this was completed, any water in the ethyl acetate layer was removed. Normally this is done by allowing the solutions to sit until any layers are completely separated, however, this could not be carried out because the Tween 80 ® added to the Dixon's Broth emulsified with the water. Instead the solution was centrifuged at 4 000 rpm for 3 hours then the water layer was removed.
4. Magnesium sulphate was added to the extraction, proportional to the amount of water visible then the ethyl acetate was removed.
5. The ethyl acetate extraction was then rotary evaporated to remove the ethyl acetate and leave any extract. The flask was washed three times in ethyl acetate and the solution placed into a pre weighed vial.
6. The vial was vacuum centrifuged to remove all the ethyl acetate then the vial was weighed to obtain the amount of extract produced.
7. The extract was brought up to a concentration of 10 mg/ml using 100 % methanol. The volume of methanol added to the vial was determined by the following: Initial weight of vial divided by the final weight of vial (X)
8. The weight of the extract (X) needs to be brought up to a concentration of 10 μg/ml

eg: X = 130.0 μg need to add 13 ml to make up to 10 μg/ml.

9. The extract was then sent to Gill Ellis, Marine Chemistry Group, University of Canterbury and was assayed against the following microbes; *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, C.albicans, Trichophyton*

mentarophytes, Cladosporium resinae, and Herpes Simplex Virus and/or Polio Virus Type 1 (inoculated in mouse kidney BSC-1 cells).

3. Results

3.1 Culture

3.1.1 Culturing of Freeze-Dried Isolate

Since it is thought that *M. furfur* is thought to be difficult to grow, a positive result here was the acquisition of cultures. All three methods proved to be successful in culturing *M. furfur* from the freeze-dried form. The addition of freeze-dried *M. furfur* to Dixon's Broth and incubation for 24 hours proved to be the most successful. Dixon's Medium proved to be very effective as a growth medium and was chosen as the base medium for this thesis.

3.1.2 Culturing of Skin Isolate

The rehydrated skin scraping produced excellent cultures of *M. furfur* after 24 hours. The dehydrated skin placed directly onto Dixon's Medium showed *M. furfur* colonies surrounding the skin after approximately 10 days.

3.2 Identification methods for *Malassezia furfur*

M. furfur grew well on SDA amended with olive oil. *M. furfur* growth on normal SDA was noted, however, this may have been due to residual nutrients from the inoculum broth. These cultures were left for a further 5 days to assess further growth. No substantial growth was obtained from SDA. Christensen's Urea Agar gave the characteristic positive reaction of a pink colour showing that *M. furfur* hydrolyses urea (Figure 3:1). These results, along with morphological and physiological characteristics, confirmed that the organism supplied by CDC is *Malassezia furfur*.

3.3 Growth Media

3.3.1 Media Incorporating Indicators

3.3.1.1 Utilisation of Urea

Investigations into commercially available medum for a positive identification of *M. furfur* proved useful (Table 3:1). Christensen's Urea Agar gave the distinctive positive result when inoculated with *M. furfur* and *R. glutinis* cultures. Phenol Red Agar gave a positive result of a colour change from orange to pink with *C. albicans*. With Urea Agar *M. furfur* and *R. glutinis* gave a positive result and Dixon's Agar with Christensen's Urea Salts, *M. furfur* gave the distinct pink colour change quite quickly whilst *R. glutinis* gave a positive result after 72 hours. Dixon's Agar is dark orange/brown in colour thus making the observation of the colour change from pink to orange difficult.

These results do not enable the development of a specific medium from commercially available media, however, it does indicate that *M. furfur* can grow on conventional media if a lipid source is added.

Table 3:1 Media Indicating Urea Utilisation

	Phenol Red Agar	Urea Agar	Dixon's with Urea
C. albicans	**Positive**	Negative	Negative
C. parapsilosis	Negative	Negative	Negative
M. furfur	Negative	**Positive**	**Positive**
R. glutinis	Negative	**Positive**	**Positive**
R. rubra	Negative	Negative	Negative
S. carlsbergensis	Negative	Negative	Negative
S. cerevisiae	Negative	Negative	Negative

Key: Positive - Colour change from orange to pink

Negative - No colour change

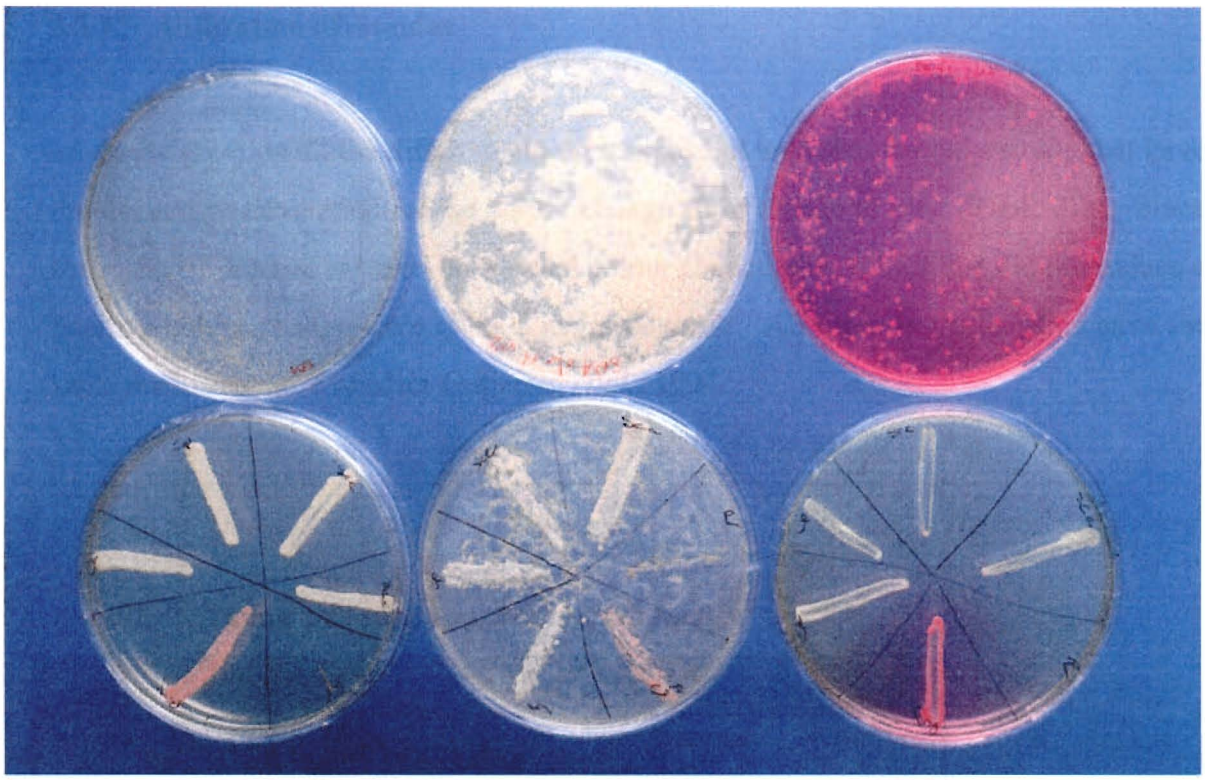


Figure 3:1 Indicator Media:
Top: *Malassezia furfur* inoculated on SDA, SDA amended with olive oil and Christensen's Urea Agar.
Bottom: Control Yeasts on above media
Clockwise from bottom, *R. glutinis, C. albicans, C. parapsilosis S. carlsbergensis, S. cerevisiae* and *R. rubra*

3.3.1.2 Utilisation of Nitrogen Sources

Further investigations into nutrient requirements were investigated with reference to special nitrogen sources. All organisms grew on this medium but only *M. furfur* and *C. albicans* gave a positive result of a colour change from green to purple (Table 3:3).

3.3.1.3 Utilisation of Amides

M. furfur grew on all the different amide sources but urea was the only amide that gave the desired positive result of the colour change from orange to pink (Table 3:2). Since the result of this test is based on a visual colour change, the amides that did not induce a positive colour change were given a negative result. All other test organisms gave the same results as those of Mira-Gutierrez *et al.*, 1994.

Table 3:2 Amide Utilisation by *Malassezia furfur*

Amide	Result	Amide	Result
Acetamide	Negative	L-phenylalaninamide	Negative
Allylurea	Negative	Propionamide	Negative
Benzamide	Negative	Salicylamide	Negative
2-chloracetamide	Negative	Thioacetamide	Negative
Formamide	Negative	Thiourea	Negative
Iso-nicotinamide	Negative	Urea	**Positive**
Malonamide	Negative	Control	Negative

Key: Positive – Colour change from orange to pink

Negative – No colour change

This result reiterated that *M. furfur* hydrolyses urea but it is disappointing that other amides were not hydrolysed as this limits further research into this area. There were signs of *M. furfur* growth on all amides but this was not considered a positive result because no colour change was observed.

Table 3:3 Nitrogen Utilisation

	Creatine			Ammonium	Acetate		Aspartic	Acid	
	1%	3%	5%	1%	3%	5%	1%	3%	5%
C. albicans	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
C. parapsilosis	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
M. furfur	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
R. glutinis	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
R. rubra	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
S. carlsbergensis	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
S. cerevisiae	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Key: Positive - colour change from green to purple

Negative - no colour change

3.3.2 Media Incorporating Lipids

3.3.2.1 Utilisation of Polyoxyethylene Sorbitan Esters (Tween®)

Tween® specificity was investigated using Dixon's Agar amended with differing concentrations of Tweens®. The results indicated that *M. furfur* can utilise all three Tweens® up to 20% concentration (Table 3:4).

Table 3:4 Lipid Utilisation of *Malassezia furfur*

	Tween 20®	Tween 40®	Tween 80®	Glycerol
1%	+++	+++	+++	++
3%	+++	+++	+++	++
5%	+++	+++	+++	++
10%	+++	+++	+++	++
15%	+++	+++	+++	++
20%	+++	+++	+++	++

Key: +++ excellent growth
++ good growth

The 24 hour broth cultures proved to have a too high cell concentration with all plates having over 300 colonies per plate. Glycerol also sustained *M. furfur* growth but not as effectively as any of the other lipids tested.

3.3.2.2 Utilisation of Cholesterol, Milk and Egg Yolk

Cholesterol Agar assessed did not sustain *M. furfur* growth. The cholesterol agar only sustained *R. glutinis* growth, indicating a potential selective medium for *R. glutinis*.

Bromocresol Purple Milk Agar sustained growth of *C. albicans* only. The positive result of a colour change from green to purple was also seen by *C. albicans*. Some small colonies of *M. furfur* did appear after 4 days incubation which did change colour, however, the too few colonies and length of incubation time limits this medium for rapid diagnosis.

Egg Yolk Agar sustained excellent growth for all yeasts. This medium is not a candidate for the selective medium targeted (Table 3:5).

Table 3:5 Utilisation of Cholesterol Agar, Egg Yolk Agar and Bromocresol Purple Milk Agar.

	Cholesterol Agar	Bromocresol Purple Milk Agar	Egg Yolk Agar
C. albicans	Negative	**Positive (Colour change)**	**Positive**
C. parapsilosis	Negative	Negative	**Positive**
M. furfur	Negative	**Positive (colour change)**	**Positive**
R. glutinis	**Positive**	Negative	**Positive**
R. rubra	Negative	Negative	**Positive**
S. carlsbergensis	Negative	Negative	**Positive**
S. cerevisiae	Negative	Negative	**Positive**

Key: Positive - Growth
Negative - No growth

3.3.3 Media Incorporating Other Nutrients

3.3.3.1 Growth on Sweat Medium

Murphy's Sweat Medium contains a great variety of nutrients at low concentrations (See Appendix I). This medium sustained growth for all yeasts therefore making if redundant as a specific medium for *M. furfur*. However, some of the nutrients that have been investigated in this work are found in Murphy's Sweat Medium (creatine and urea).

3.3.3.2 Utilisation of Taurocholic Acid

Omission of bile from Dixon's Agar limited growth of *M. furfur* (Figure 3:2). The various concentrations of taurocholic acid all gave the same amount of growth, thus indicating that the lowest concentration of taurocholic acid is sufficent to sustain *M furfur* growth. It is interesting to note that the higher concentrations do not have any inhibitory effect on the growth of *M. furfur* (Figure 3:3). The control medium, which contained no taurocholic acid or bile, sustained little *M. furfur* growth. This indicates that the removal of bile salts from Dixon's Agar significantly reduces the growth rate of *M. furfur*. Therefore, for optimum growth the addition of taurocholic acid is essential.

Table 3:6 Taurocholic Acid Utilisation

	0.5 %	1.0 %	1.5 %	2.0 %	2.5 %	Control
M. furfur	+++	+++	+++	+++	+++	–

Key: +++ Excellent Growth

– No Growth

Figure 3:2 No Taurocholic Acid or Oxgall (left) and Dixon's Agar (right)

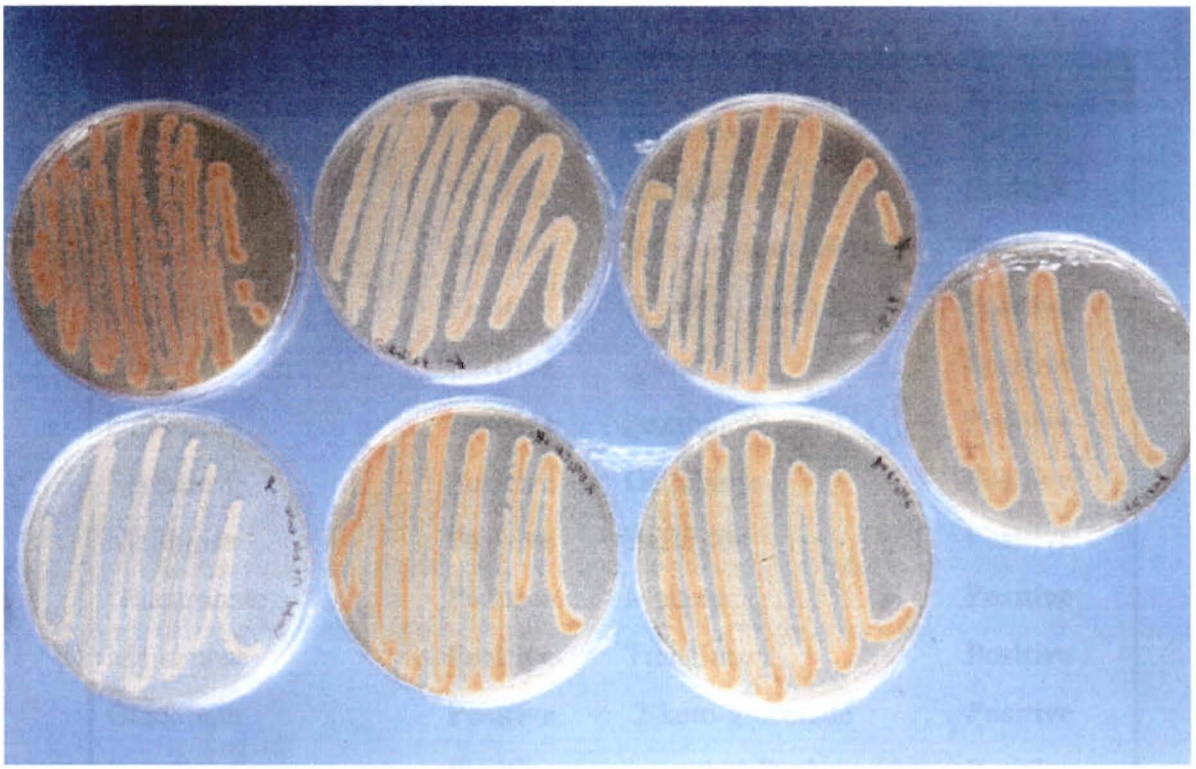


Figure 3:3 Taurocholic Acid Assimilation:
Top left (clockwise) Dixon's Agar, 0.5%, 1.0%, 1.5%, 2.0% & 2.5% Taurocholic Acid, plus control

3.3.3.3 Assessment of ID 32C Substrate Assimilation System

Following the set protocol for the identification system it was found that *M. furfur* did not grow. The comparison yeasts, however, gave the expected results and were analysed by Nicola Egerton, Medbio Enterprises Limited, Christchurch to confirm the identification. The addition of Tween 80® and glycerol to the suspension medium (See Appendix II) gave some positive findings (Table 3:7). These results indicate that *M. furfur* can utilise a variety of substrates provided a sufficient lipid source is present.

Table 3:7 Substrate Identification System

Substrate	*M. furfur*	Substrate	*M. furfur*
Sorbitol	**Positive**	Galactose	**Positive**
D-xylose	**Positive**	Actidione	**Positive**
Ribose	**Positive**	Sucrose (saccharose)	**Positive**
Glycerol	**Positive**	N-acetyl-glucosamine	**Positive**
Rhamnose	**Positive**	$_{DL}$-lactate	**Positive**
Palatinose	**Positive**	$_{L}$-arabinose	**Positive**
Erythritol	**Positive**	Cellobiose	**Positive**
Melibiose	**Positive**	Raffinose	**Positive**
Glucuronate	**Positive**	Maltose	**Positive**
Melezitose	**Positive**	Trehalose	**Positive**
Gluconate	**Positive**	2-keto-gluconate	**Positive**
Levulinate	**Positive**	α-methyl-D-glucoside	**Positive**
Glucose	**Positive**	Mannitol	**Positive**
Sorbose	**Positive**	Lactose	**Positive**
Glucosamine	**Positive**	Inositol	**Negative**
Esculin	**Positive**	Control	**Positive**

Key: Positive – opaque wells in test strip Negative – wells stay clear

3.3.3.4 Gelatine Hydrolysis

The result of the hydrolysis (Table 3:8) of gelatine by *M. furfur* and comparison organisms concurred with the results discussed by Petruccioli and Gallo-Federici (1992)

Table 3:8 Gelatine Hydrolase activity of *Malassezia furfur*

Yeast	Gelatine Hydrolase Activity
C. albicans	Positive
C. parapsilosis	Negative
M. furfur	Weak
R. glutinis	Positive
R. rubra	Negative
S. carlsbergensis	Positive
S. cerevisiae	Weak

Key: Positive – complete liquefaction of medium
Weak – partial liquefaction of media
Negative – no liquefaction of medium

During the course of this work, various media were inoculated with *M. furfur* to assess sustainable growth. The following media were all supplemented with 10 % Tween 80 ® and 2.5 % glycerol unless the medium already contained them or had an alternative lipid source present (*). Those media that did not sustain *M. furfur* growth are highlighted.

Table 3:9 Summary of Media Investigated

Medium	Growth of *M. furfur*	Medium	Growth of *M. furfur*
Bromocresol Purple Agar	Good	SDA with olive oil	Satisfactory
Bromocresol Purple Milk	Poor	SDA with Tween 80® and	Good
Yeast Extract Agar *		glycerol	
Cholesterol Agar *	Poor	Sweat Medium *	Satisfactory
Christensen's Urea Agar	Good	Tween 80® Medium II *	Satisfactory
Dixon's Agar *	Excellent	Tween 80® Medium *	Satisfactory
Egg Yolk Agar *	Good	Urea Agar Base	Good
Gelatine agar	Poor	Yeast Extract Agar	Good
Lipid Agar *	Poor	Yeast Extract Malt Extract	Good
		Agar	
Phenol Red Broth Base	Satisfactory		

3.4 Induction of the Hyphal Phase of *Malassezia furfur*

As previously mentioned, *M. furfur* did not grow on the Cholesterol Agar, therefore, hyphal induction was not possible. *C. albicans* gave excellent hyphal production in human sera but *M. furfur* did not.

3.5 Reaction to Inhibitory Agents

3.5.1 Antifungal Agents

3.5.1.1 Amphotericin B Sensitivity

The test organisms were resistant to amphotericin B at all concentrations (Table 3:9) however, *A. fumigatus* was susceptible to amphotericin B indicating that the amphotericin B supplied was active. Fungal resistance to amphotericin B is rare but is possible. Those fungi and yeasts that are resistant to amphotericin B lack ergosterol in the cell wall (McGinnis and Rinaldi, 1991).

The amphotericin B assay gave no zones of inhibition therefore no table of results are given here. This may be due to the inability of amphotericin B to diffuse through the medum or that amphotericin B is fungistatic not fungicidal. A too high concentration of yeast inoculum would reduce the effectivity of amphotericin B.

Table 3:9 Sensitivity to Amphotericin B in Agar Dilution Series

	5.1 mg/ml	2.5 mg/ml	1 mg/ml
A. fumigatus	_	_	_
A. niger	_	_	_
F. oxysporum	_	_	_
C. albicans	+++	+++	+++
C. parapsilosis	+++	+++	+++
M. furfur	+++	+++	+++
R. glutinis	+++	+++	+++
R. rubra	nt	nt	Nt
S. carlsbergensis	+++	+++	+++
S. cerevisiae	+++	+++	+++

Key: +++ Resistant _ Susceptible nt Not tested

These results should be treated with scepticism because all the fungi tested should have been sensitive to amphotericin B. A sample of *C. albicans* strain used in this experimental work has been sent away for sensitivity testing to amphotericin B.

3.5.1.2 Nystatin Sensitivity

All yeasts were susceptible to Nystatin at the lowest concentration of 1 mg/ml (5060 U/ml) (Table 3:10). A positive control was difficult to nominate, as the cases of fungal resistant to Nystatin are rare (McGinnes and Rinaldi, 1991).

Table 3:10 Sensitivity to Nystatin in the Agar Dilution Series

	5 mg/ml	2.5 mg/ml	1 mg/ml
C. albicans	–	–	–
C. parapsilosis	–	–	–
M. furfur	–	–	–
R. glutinis	–	–	–
R. rubra	–	–	–
S. carlsbergensis	–	–	–
S. cerevisiae	–	–	–

Key – Susceptible

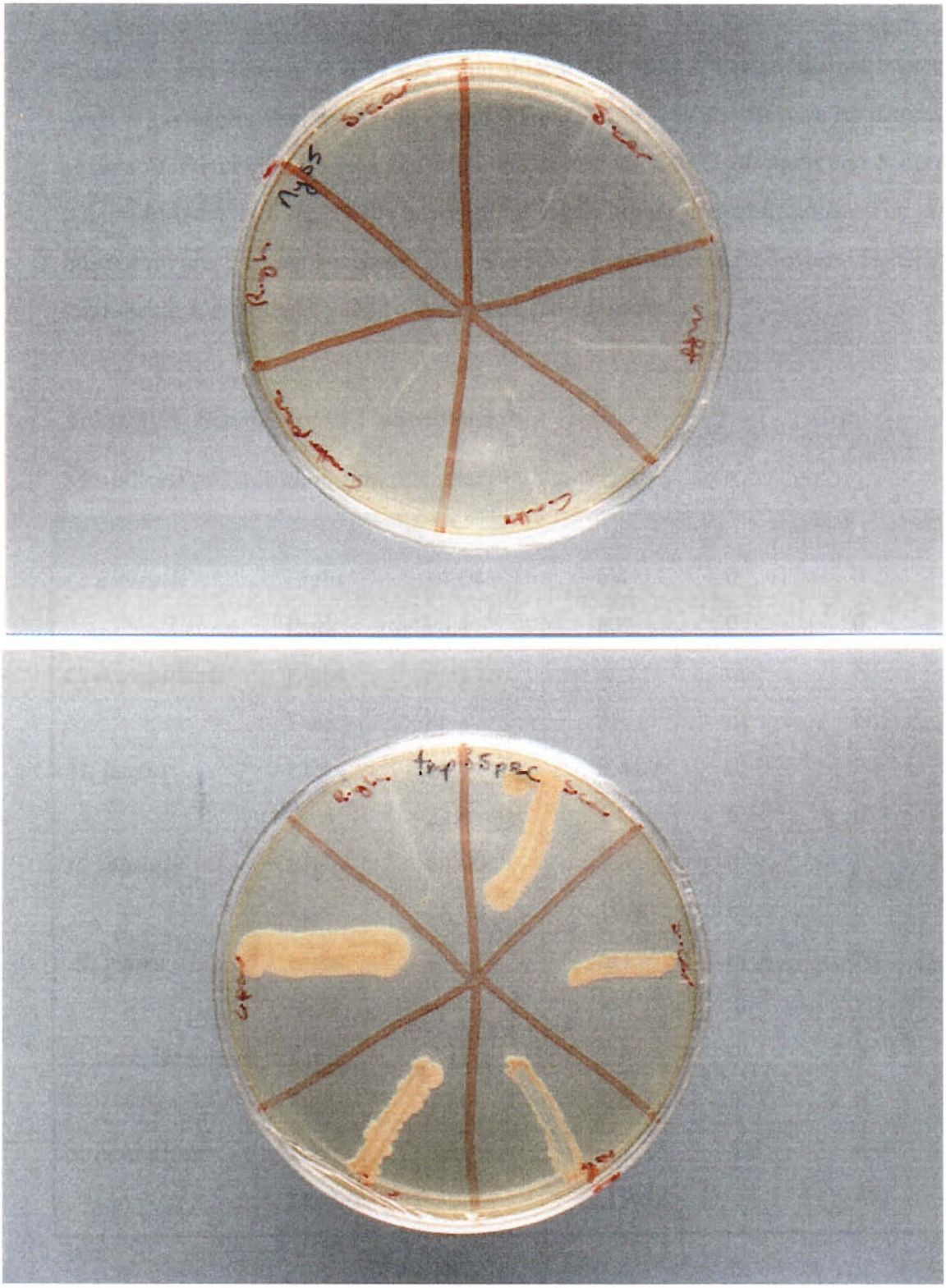


Figure 3:4 Nystatin (top) and Amphotericin B (bottom) Sensitivity test

3.5.1.3 Thiarubrine A Sensitivity

Constable and Towers (1989) state that thiarubrine A is effective against bacteria and fungi at concentrations as low as 1 ppm. Thiarubrine A is an effective antifungal agent against *M.* furfur (Figure 3:5a and 3:5b) when compared to *C. albicans* and *S. cerevisiae* (Constable and Towers, 1989) however, at much higher concentrations. The zones of inhibition are similar to those published by Constable and Towers (1989). The thiarubrine A supplied by Dr Towers was a pure sample.

Table 3:11 Sensitivity to Thiarubrine A

Mean Zone of Inhibition (mm diameter)

		10^{0}	10^{-1}	10^{-2}	10^{-3}
C. albicans	Light	14.64	6.4	0	0
	Dark	16.4	8.8	0	0
C. parapsilosis	Light	nt	nt	nt	Nt
	Dark	nt	nt	nt	Nt
M. furfur	Light	16.44	8.44	0	0
	Dark	22.0	14.56	6.68	0
R. glutinis	Light	16.00	3.0	0	0
	Dark	18.00	10.83	0	0
R. rubra	Light	nt	nt	nt	Nt
	Dark	nt	nt	nt	Nt
S. carlsbergensis	Light	9.12	2.0	0	0
	Dark	12.0	4.0	0	0
S. cerevisiae	Light	26.9	17.28	8.0	6.0
	Dark	38.4	19.52	11.1	8.0

Key: nt - not tested

Figure 3:5a Thiarubrine A (light)

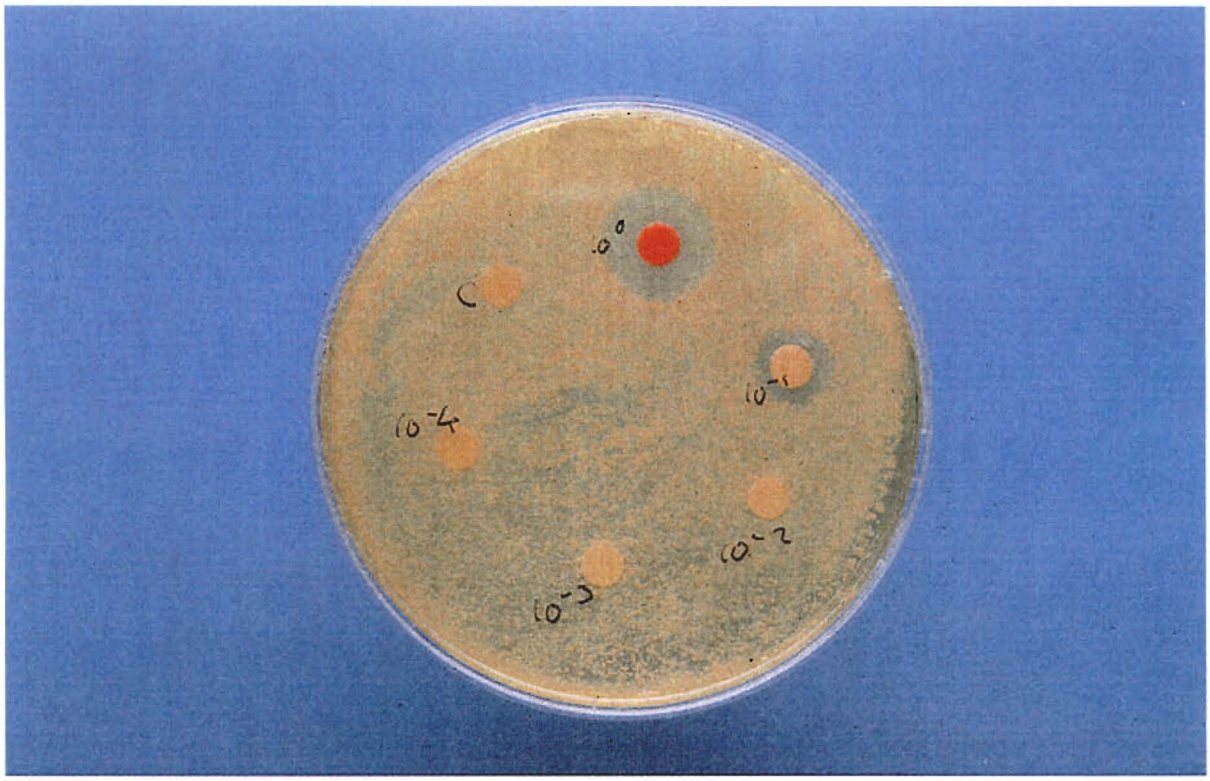


Figure 3:5b Thiarubrine A

3.5.1.4 Agricultural Fungicide Sensitivity

Copper oxychloride (Copper - metal), Captan (Sulfenimide), and Manzeb (Dithiocarbamate) were shown to slightly inhibit *M. furfur* growth whist Alleate (Fosethyl-aluminium), Bravo (Chlorothalonil nitrate), Greenguard (Chlorothalonil and thiophanate-methyl), Saprol (Triforine (piperazine) and Zineb (Dithiocarbamate) had no effect on *M. furfur* (Table 3:12). *C. albicans, C. parapsilosis, S. carlsbergensis* and *S. cerevisiae* were not effected by any of the antifungal agents, whilst *R. glutinis* and *R. rubra* were mildly inhibited by all antifungal agents

Table 3:12 Agricultural Fungicide Sensitivity

	Alleate	Bravo	Captan	Copper oxychloride	Green guard	Manzeb	Saprol	Zineb
M. furfur	+++	++	++	++	+++	++	+++	+++
C. albicans	+++	+++	+++	+++	+++	+++	+++	+++
C. parapsilosis	+++	+++	+++	+++	+++	+++	+++	+++
S. carlsbergensis	+++	+++	+++	+++	+++	+++	+++	++
S. cerevisiae	+++	+++	+++	+++	+++	+++	+++	+++
R. glutinis	++	++	++	++	++	++	++	++
R. rubra	++	++	++	++	++	++	++	++

Key: +++ Resistant, ++ Moderately resistant

3.5.2 Effect of Fungal Cell Wall Degrading Enzymes

The most effective enzyme appears to be α-mannosidase which successfully lysed all yeasts. *C. albicans* and *C. parapsilosis* were resistant to all other enzymes, whilst *S. carlsbergensis* was moderately resistant to all other enzymes. *M. furfur* was resistant to Lyticase, moderately resistant to Driselase and Lysozyme but was moderately susceptible to β-glucosidase, and susceptible to α-mannosidase and Novozyme. (Table 3:13).

The β 1-3 glucanase assay showed no inhibition of *C. albicans*, *S. carlsbergensis* or *M. furfur* at the concentrations tested This may be due to either a too high concentration of cells or a too low concentration of enzyme.

Table 3:13 Effect of Fungal Cell Wall Degrading Enzymes

	Drislase	β Glucosidase	Lyticase	Lysozyme	α Mannosidase	Novozyme
C. albicans	++	+++	+++	+++	_	+++
C. parapsilosis	+++	+++	+++	+++	_	+++
M. furfur	++	+	+++	++	_	_
R. glutinis	+	+	_	_	_	_
R. rubra	nt	nt	nt	nt	Nt	nt
S. carlsbergensis	++	++	++	++	_	++
S. cerevisiae	+	+	+	+	_	+

Key: +++ Resistant, ++ moderately resistant, + moderately susceptible, _ susceptible, nt Not tested

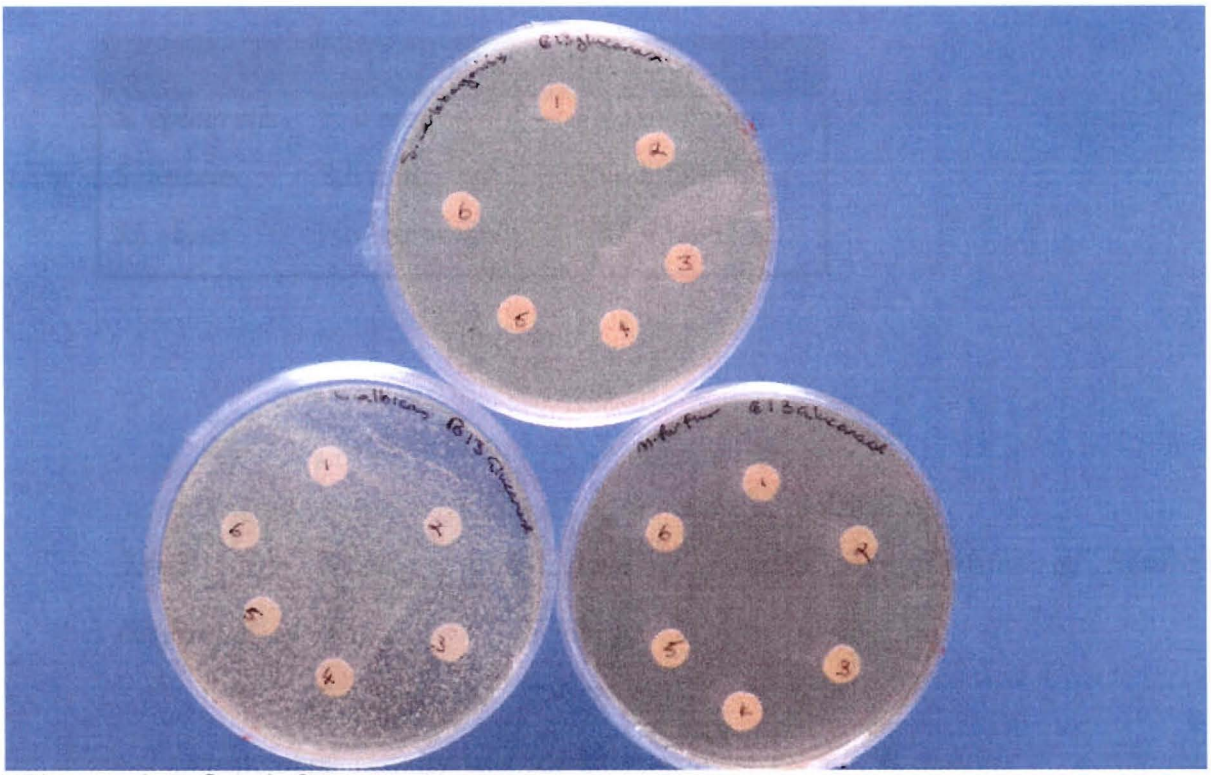


Figure 3:7 β 1-3 Gluconase Assay

3.6 Antimicrobial agents produced by *Malassezia furfur*

3.6.1 Azelaic Acid Assay

S. epidermis, and *S. aureus* were susceptible to 20% azelaic acid but *M. canis* was resistant. No test organisms were susceptible to *M. furfur*.

Table 3:14 Azelaic Acid Assay

	Azelaic Acid	M. furfur
S. epidermis	5. 0 mm	No inhibition
S. aureus	8.0 mm	No inhibition
M. canis	No inhibition	No inhibition

3.6.2 Ethyl Acetate Extraction of Fungal Metabolites of Malassezia furfur

No antimicrobial activity was identified against *E. coli, B. subtilis, P. aeruginosa, C. albicans, T. mentarophytes, C. resinae*, and Herpes Simplex Virus and/or Polio Virus Type 1. The production of azelaic acid by *M. furfur* may be slow, Nassaro-Porro and Passi (1978) grew their cultures for 30 days before extracting any compounds. The protocols used here may not be suitable for extraction of azelaic acid.

4. DISCUSSION

4.1 Culture and Growth

The current work has shown that Dixon's Agar contains all appropriate nutrients for *M. furfur* growth. There is the perception that *M. furfur* is difficult to culture but this work had no difficulties in obtaining and maintaining *M. furfur* cultures on this medium. This work showed that SDA amended with olive oil was difficult to work with as the medium underwent syneresis, that is the olive oil came out of the medium and leaked out of the plates when incubated. Olive oil is quite volatile and when platinum loops and glass spreaders were flame sterilised the oil would burst into flames covering the hand. SDA amended with olive oil is not recommended for culturing *M. furfur* isolates. Christensen's Urea Agar also sustains *M. furfur* growth however, not as well as Dixon's Agar. *M. furfur* has weak gelatinase activity, therefore this work concurred with that Petruccioli and Gallo-Federici (1992). However, this information does not enable the creation of a selective medium for *M. furfur*.

The current work also indicated that viable cultures from *M. furfur* could be obtained from dead skin infected with *M. furfur*. These results concurred with Vollekova (1992) who commented that the viability of *M. furfur* in skin scrapings decreases over a period of three weeks but after 15 weeks colonies were still isolated from dead skin. The viability of *M. furfur* in dead skin may be related to external influences such as cold temperatures and lack of moisture (Vollekova, 1992). If *M. furfur* is sloughed off with dead skin cells there is a possibility that *M. furfur* infections could be transferable. Since *M. furfur* is commonly found on human skin, awareness may only be needed in clinical institutions when dealing with the gravely ill such as intensive care neonates and immunocompromised patients.

4.2 Nutrient Requirements

It has been well documented that *M. furfur* hydrolyses urea (Guého and Meyer, 1989; Klotz, 1989; Marcon and Powell, 1992). This work has shown that Christensen's Urea Agar amended with a lipid source gives a rapid colour change which could be useful in the identificaiton of *M. furfur*. However, Dixon's Agar amended with Christensen's Urea Salt Solution added gave the positive result but because Dixon's Agar is a dark medium the colour change was not very visible. This work also indicated that suitable nitrogen sources for *M. furfur* include creatine or aspartic acid. Assessment of amides indicated that *M. furfur* did not give the positive result of a colour change, this may be due to a lack of specific amidase activity in *M. furfur*. These result follow those of Mira-Gutierrez *et al.* (1994) which indicated that many of the yeast species tested utilised only one or two amides due to a lack of or limited amidase activity (Mira-Gutierrez *et al.*, 1994)

Investigations of lipid utilisation were more promising. The current work indicated that *M. furfur* can utilise all three Tweens' at various concentrations. These results concurred with those of Guého *et al.* (1996) who also indicated that Tween 20® was utilised by *M. furfur* and *M. slooffiae* and but not by other *Malassezia* species. If Tween 80® was replaced with Tween 20® in Dixon's Agar this would limit the number of *Malassezia* species that would grow down to *M. furfur* and *M. slooffiae* as only these *Malassezia* species can utilise Tween 20®

The use of Cholesterol Agar did not support growth of *M. furfur*. It was hoped that this medium would induce hyphal growth *in vitro* but that was not possible. This medium did provide a suitable substrate for *R. glutinis*. The Bromocresol Purple Milk Agar did sustain slow *M.* furfur growth, which changed colour but not in a satisfactory time frame of 24 - 48 hours. Egg yolk was shown an inexpensive alternative for Tweens'. Sweat Medium could also be an alternative medium for *M. furfur* cultures, however, since the components of this medium are numerous is it difficult to identify those that are specifically utilised by *M. furfur*.

The addition of Tween 80® to the suspension medium in the ID32C Identification system gave some very pleasing results. *M. furfur* is not included in the number of yeasts that this system identifies. This is because *M. furfur* is able to use lipids as a sole carbon source so the standard sugar and fermentation tests are not carried out (Ahern and Simmons, 1997). However, the current work has indicated that *M. furfur* will assimilate a variety of sugars and carbohydrate sources provided there is the prerequisite lipid source.

Taurocholic acid (2-(((3 alpha, 5 beta, 7 alpha, 12 alpha)-3,7,12-trihydroxy-24-oxocholan-24yl)amino)ethanesulfonic acid) (Medline-Database, 1998) is a sodium salt formed from taurine and cholic acid. This acid is a major component of the bile of carnivorous animals which acts like a detergent to solubilize fats for absorption and is itself absorbed (Benmoussa *et al.*, 1993). Bile salts substituted by taurocholic acid in Dixon's Agar gave some promising results. Bile salts give the medium a very dark orange/brown colour; however, the substitution of taurocholic acid gave the medium a clear appearance and may permit the addition of a indicator into the medium and allow a colour change to be easy to visualise. This work also indicated that *M. furfur* grew very well at the lowest concentration of taurocholic acid. This result will add to the information about the nutrient requirements of *M. furfur*.

Oxbile™ has been used by Bergbrant *et al.* (1992) who assessed two different contact plates for isolation and identification of *M. furfur*. These contact plates were placed on the skin of patients to determine the ability to isolate *M. furfur* cultures directly from skin. The "Contact Plate A" was a variation of glucose/peptone agar (with Tween 80 ®), which is a common agar used in culturing of *M. furfur*, and "Contact Plate B" contained the same components with the addition of Oxbile™ (with Tween 60 ®). The "Contact Plate B" yielded over 100 x more cultures than "Contact Plate A" (Bergbrant *et al.*, 1992). This indicates that the addition of Oxbile™ (containing taurocholic acid) is essential component of any medium for *M. furfur*. Contact plates for the isolation and identification of *M. furfur* in skin conditions could be implemented as standard practice in the future.

To refine Dixon's Agar medium for more specific *M. furfur* growth, taurocholic acid should be substituted for bile salts and Tween 20® for Tween 80®. Further research is needed to determine the exact component of malt extract required by *M. furfur*. The results from this work recommends that Dixon's Agar with the addition of antibiotics replace SDA with antibiotics in clinical laboratories. The fungi and yeasts that were inoculated on Dixon's Agar during this study grew very well; therefore, there is no indication on any inhibitory effect from Dixon's Agar on fungi or yeasts. The protocols followed when quantitatively determining fungal infection can still be maintained, therefore, enabling quantitative determination of *M. furfur* infections from clinical specimens.

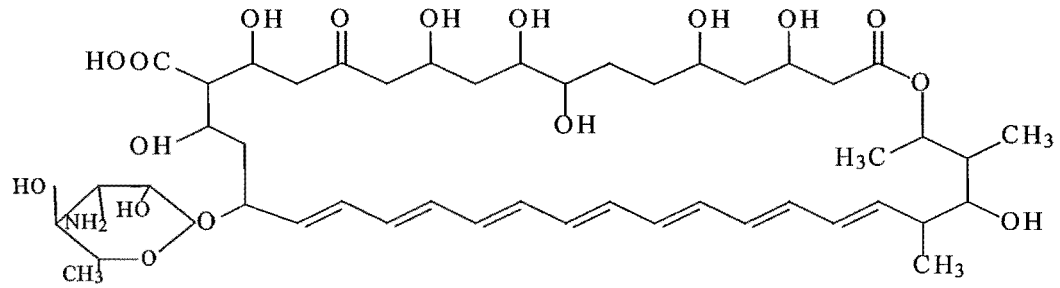
4.3 Identification and Control

Contact plates developed by Bergbrant *et al.* (1992) show promising results. The first allergenic protein of *M. furfur* has also been identified and some studies now take into account antibody production when investigating possible *M. furfur* infection (Broberg *et al.*, 1992). The ability of *M. furfur* to utilise a variety of lipid sources has been well examined. Many systems have been developed to identify the *Malassezia* genus (Mayser *et al.*, 1997; Guého and Meyer, 1989; Guillot *et al.*, 1996) which are all based on lipid assimilation specificity. It was found by Mayser *et al.* (1997) that *M. furfur* is the only member of it's genus to utilise PEG-7 glycerol monoalcarioate thus leading towards a specific medium for *M. furfur*

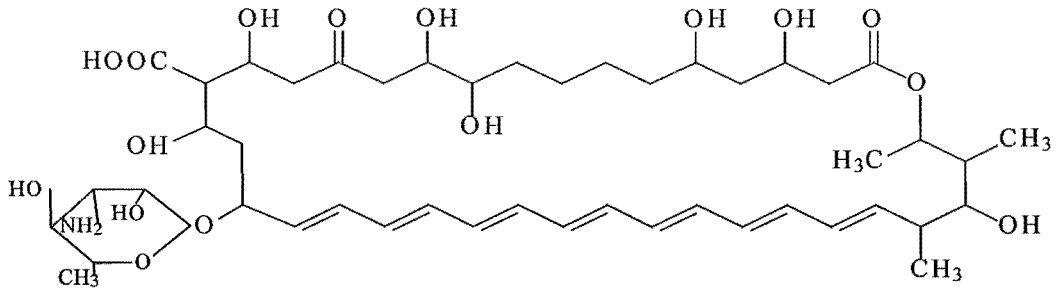
All the yeasts tested in this work were resistant to amphotericin B and the susceptible to Nystain. Amphotericin B and Nystatin resistant *Candida* spp have been reported as rare (Kerridge and Whelan, 1984; Mitsutake *et al.*, 1994) but there have been no reported cases of amphotericin B resistance in any other of the comparison yeasts used in this work. It has been reported that *M. furfur* was resistant to Nystatin (Adams, 1987), however, this work has shown that it is susceptible. This work must be looked upon

with some scepticism. The susceptibility of all tested organisms to Nystatin was to be expected, but the resistance to amphotericin B was not.

The chemical structure of amphotericin B and Nystatin are very similar (Figure 4:1) and their mode of action is also similar (Brajtburg *et al.*, 1990; Kerridge and Whelan, 1984). The susceptibility of *M. furfur* to Nystatin could lead to the prescription of Nystatin based creams for *M. furfur* skin infections, however, clinical trials will need to be completed.



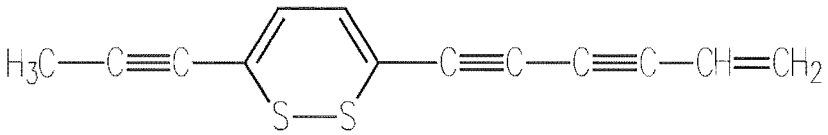
AMPHOTERICIN B



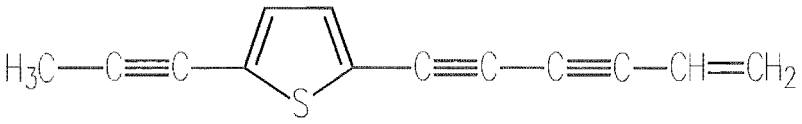
NYSTATIN

Figure 4:1 Comparative structures of Amphotericin B and Nystatin (Kerridge and Whelan, 1984)

Thiarubrine A (Figure 4:2) is a dithiacyclohexadiene polyene compound from members of the Asteraceae (*Chaenactis douglasii* and *Rudbeckia harta*) (Constable and Towers, 1989). Thiarubrine A is light sensitive and transforms to thiophene when exposed to UV-A. Thiarubrine A has proven to be a strong inhibitor of *C. albicans* and *S. cerevisiae*, and now *M. furfur*. Further, thiarubrine A appears to be more effective than thiophene against *M. furfur*. There is the possibility of using thiarubrine A as an effective antifungal agent against *M. furfur*.



Thiarubrine A



Thiophene A

Figure 4:2 Polyenes from *Chaenactis douglasii* (Constable and Towers, 1989)

Preventative treatment is recommended to preclude recurrence of *M. furfur* skin infections. Shampoos containing selenium sulphide and Coal Tar Gel are often prescribed, as well as topical applications of ketoconazole (Faergemann, 1997; Wright *et al.*, 1993). To reduce the possibility of antifungal resistant *M. furfur* strains the use of an alternative treatment should be implemented. Tea-tree oil is a naturally occurring compound that has been found effective against *M. furfur* (Nenoff *et al.*, 1996). Tea tree oil incorporated into soaps and lotions are readily available. However, a common skin condition, contact eczema, has been linked with an allergic reaction to certain compounds found in tea-tree oil (limonene, α-terpinene, aromadendrene, terpinen-4-ol, *p*-cymene and α-phellandrene) so this needs to be taken into account when recommending this oil as a prophylactic form of treatment (Knight and Hausen, 1994).

The degradation of *M. furfur* by various cell wall degrading enzymes gave positive results that require further work. Novozyme and α-mannosidase were particularly effective against *M. furfur*. Investigations into the state of the cell wall after incubation with cell wall degrading enzymes by use of the scanning electron microscope (SEM) was not possible because of prolonged repairs on the SEM. It is now known that, because of the act of unipolar budding, there is a noticeable concentration of chitin around the budding collar or scar (Ahern and Simmons, 1997). The area of chitin inhibition or degradation could be, therefore, of some interest in regards to *M. furfur* inhibition and treatment.

4.4 Future Research

The field of mycology research seems to be turning increasingly to molecular based identification techniques. These techniques (Immuno-assays, PCR or ELISA) are much quicker and accurate for identifying fungal pathogens than traditional culture methods. Future research could include the implementation of a study concerning the identification of *M. furfur* antibodies in blood thus increasing time and accuracy in fungal identification. The epidemiology of *M. furfur* has not been thoroughly assessed. This work has shown that *M. furfur* infections are transferable, therefore patients with

severe skin injuries, such as burns, could be at risk. To date, no research has been completed on the possibility of identifying *M. furfur* infections in the wounds of burn patients. An investigation into the identification of *M. furfur* by using the ELISA-Oligonucleotide Protocol is also an are of interest but was not possible due to lack of equipment and funding. This system gives a rapid visual identification or many microbes and could be a excellent technique for identifying either the *Malassezia* genus or *M. furfur*

4.5 SUMMARY

M. furfur causes the skin infection pityriasis versicolor, *Malassezia* folliculitits and seborrhoeic dermatitis also deeper sepsis has also been identified. It is thought that *M. furfur* is difficult to culture but this work has found culturing of *M. furfur* to be relatively uncomplicated. This is due to Dixon's Agar, which was an excellent medium for *M. furfur*. Implementation of Dixon's Agar amended with antibiotics in clinical laboratories and research facilities would be a more suitable medium for culturing *M. furfur*. Dixon's Agar could be improved further by replacing bile salts with taurocholic acid and Tween 40® with PEG-7 glycerol monoalcarioate as a lipid source.

The preventative treatment of *M. furfur* with tea-tree oil is an option instead of synthetic antifungal agents. Thiarubrine A has also been found as an effective antifungal agent against *M. furfur*. The degradation of *M. furfur* cell wall by Novozyme needs to be clarified since the exact composition of Novozyme is not known.

Further work is always needed on organisms such as *M. furfur*. This work has shown that *M. furfur* is not difficult to culture, therefore, correct identification of this yeast will be possible. Thus the prescription of the correct antifungal agent can be given.

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6. Appendix of Media

6.1 Dixon's Agar (Van-Gerven and Odds, 1995)

Composition per litre:

Malt extract (Oxoid)	40.0 g
Oxgall (Oxoid) or Bile (Sigma)	20.0 g
Agar (Gibco)	20.0 g
Tween 40® (Sigma)	10.0 ml
Glycerol (BDH, AnaLar)	2.5 ml

Preparation of medium: Add all components to 1.0 l distilled water. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115 ° C. Pour into sterile petri dishes.

6.2 Dixon's Broth

Composition per litre:

Malt extract	40.0 g
Oxgall or Bile	20.0 g
Tween 40®	10.0 ml
Glycerol	2.5 ml

Preparation of medium: Add all components to 1.0 l distilled water. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115 ° C. Pour into sterile universal bottles.

6.3 Dixon's Agar with Cycloheximide, Chloramphenicol and Gentamicin (CCG)

Composition per litre:

Malt extract	40.0 g
Oxgall or Bile	20.0 g
Agar	20.0 g
Tween 40®	10.0 ml
Glycerol	2.5 ml
Cycloheximide solution	10.0 ml
Chloramphenicol solution	10.0 ml
Gentamicin solution	10.0 ml

pH 5.6 ± 0.2 at 25°C

Cycloheximide solution:

Composition per 10.0 ml

Cycloheximide (Sigma)	0.5 g
Acetone (BDH, AnaLar)	10.0 ml

Preparation of Cycloheximide solution: Add Cycloheximide to acetone. Mix thoroughly.

Chloramphenicol solution

Composition per 10.0 ml

Chloramphenicol (Sigma)	0.5 ml
95% Ethanol (BDH, AnaLar)	10.0 ml

Preparation of Chloramphenicol solution: Add chloramphenicol to ethanol. Mix thoroughly.

Gentamicin solution

Composition per 10.0 ml

Gentamicin	0.5 g
95% Ethanol	10.0 ml

Preparation of Gentamicin solution: Add gentamicin to ethanol and mix well. Filter sterilise.

Preparation of medium: Add all components to except Cycloheximide, Chloramphenicol and Gentamicin solutions to 1.0 l distilled water. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115°C. Aseptically add the Cycloheximide, Chloramphenicol and Gentamicin solutions to the cooled medium. Mix thoroughly. Pour into sterile petri dishes.

6.4 Sabouraud Dextrose Agar (SDA) (Atlas, 1993)

Composition per litre:

Glucose	40.0 g
Agar	15.0 g
Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g

pH 5.6 ± 0.2 at 25°C

Source: Available premixed through BBL Microbiology systems

Preparation of medium: Add all components to distilled water and bring volume to 1.0 l. Mix thoroughly. Autoclave for 15 minutes at 15 psi pressure-121 ° C. Pour into sterile petri dishes.

6.5 SDA with olive oil (Atlas, 1993)

Composition per litre:

Sabouraud Dextrose Agar	65.0 g
Olive Oil (Rizzoli)	10.0 ml

pH 5.6 ± 0.2 at 25°C

Preparation of medium: Add all components to distilled water and bring volume to 1.0 l. Mix thoroughly. Autoclave for 15 minutes at 15 psi pressure-121 ° C. Pour into sterile petri dishes.

6.6 SDA with Tween 80® and Glycerol

Composition per litre:

Sabouraud Dextrose Agar	65.0 g
Tween 80®	10.0 ml
Glycerol	2.5 ml

pH 5.6 ± 0.2 at 25°C

Preparation of medium: Add all components to distilled water and bring volume to 1.0 l. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115 ° C. Pour into sterile petri dishes.

6.7 SDA with Tween 80®, Glycerol and CCG

Composition per litre:

Sabouraud Dextrose Agar	69.0 g
Tween 80®	10.0 ml
Cycloheximide solution	10.0 ml
Chloramphenicol solution	10.0 ml
Gentamicin solution	10.0 ml
Glycerol	2.5 ml

pH 5.6 ± 0.2 at 25°C

Cycloheximide solution:

Composition per 10.0 ml

Cycloheximide	0.5 g
Acetone	10.0 ml

Preparation of Cycloheximide solution: Add Cycloheximide to acetone. Mix thoroughly.

Chloramphenicol solution

Composition per 10.0 ml

Chloramphenicol	0.5 ml
95% Ethanol	10.0 ml

Preparation of Chloramphenicol solution: Add chloramphenicol to ethanol. Mix thoroughly.

Gentamicin solution

Composition per 10.0 ml

Gentamicin	0.5 g
95% Ethanol	10.0 ml

Preparation of Gentamicin solution: Add gentamicin to ethanol and mix well. Filter sterilise.

Preparation of Medium: Add all components except cycloheximide, chloramphenicol and gentamicin solution to distilled water and bring up to 970.0 ml. Mix thoroughly. Gently heat and bring to boiling. Autoclave for 10 minutes at 15 psi pressure-115 ° C. Allow to cool to 45°-50°C. Aseptically add the cycloheximide, chloramphenicol solution and gentamicin solution to cooled medium. Mix thoroughly. Pour into sterile petri dishes.

6.8 Christensen's Urea Agar (Atlas, 1993)

Composition per litre:

Christensen's Urea Salt Solution (CUSS)	100.00 ml
Agar	15.0 g

Christensen's Urea Salt Solution

Composition per 100 ml

Urea (BDH, AnaLar)	20.0 g
NaCl (BDH, AnaLar)	5.0 g
KH_2PO_4 (Unilab)	2.0 g
Peptone (Gibco)	1.0 g
Glucose	1.0 g
Phenol Red (BDH, Industrial Dyes)	0.012 g

Preparation of Christensen's Urea Salt Solution

Add all components to distilled water and bring up to 100 ml. Mix thoroughly. Filter sterilise.

Preparation of Medium: Add all components except Agar and bring up to 900 ml. Autoclave for 20 minutes at 15 psi pressure-121°C. Cool to 50°C. Aseptically add the 100.0 ml CUSS. Mix thoroughly and pour into sterile vessels.

6.9 Christensen's Urea Agar with Tween 80® and Glycerol

Composition per litre:

CUSS	100.00 ml
Agar	15.00 g
Tween 80 ®	10.0 ml
glycerol	2.5 ml

Preparation of Medium: Add all components except CUSS distilled water and bring up to 900 ml. Autoclave for 15 minutes at 15 psi pressure-115°C Cool to 50°C. Aseptically add the 100.0 ml CUSS. Mix thoroughly and pour into sterile petri dishes.

6.10 Urea Agar Base (Atlas, 1993)

Composition per litre:

Agar	15.0 g
NaCl	5.0 g
Na_2HPO_4	1.2g
Peptone	1.0 g
Glucose	1.0 g
K_2HPO_4	1.2 g
Phenol red	0.012 g
Urea solution	50.0 ml

pH 6.8±0.2 at 25°C

Urea solution

Composition per 100.0 ml

Urea	40.0 g

Preparation of Urea solution: Add urea to distilled water and bring volume to 100.0 ml. Mix thoroughly. Filter sterilise.

Preparation of Medium: Add components, except urea solution, to distilled water and bring volume to 950.0 ml. Mix thoroughly. Gently bring to the boiling. Autoclave for 20 minutes at 10 psi pressure-115°C. Cool to 50°C. Aseptically add 50.0 ml of sterile urea solution. Mix thoroughly. Aseptically pour into sterile petri dishes or slants.

6.11 Phenol Red Broth Base (Atlas, 1993)

Composition per litre:

Agar	15.0 g
Protease-peptone (Gibco)	10.0 g
NaCl	5.0 g
Bacto beef extract (Gibco)	1.0 g
Phenol red	0.025 g
Carbohydrate solution	20.0 ml

pH 7.4±0.2 at 25 ° C

Carbohydrate solution:

Composition per 20.0 ml:

Tween 80 ®	15.0 ml
Glycerol	2.5 ml

Preparation of Carbohydrate Solution: Add carbohydrate to distilled water and bring volume to 20.0 ml and autoclave for 10 minutes at 15 psi pressure-115°C.

Preparation of Medium: Add components, except carbohydrate solution, to distilled water and bring volume to 980.0 ml. Mix thoroughly. Adjust pH to 7.4 if necessary. Autoclave for 15 minutes at 15 psi pressure-121°C. Cool to 45° - 50°C. Aseptically add 20.0 ml sterile carbohydrate solution. Mix thoroughly. Pour into sterile petri dishes or slants.

6.12 Cholesterol Medium (Atlas, 1993)

For this medium, unless otherwise specified, all chemicals are from BDH "AnaLar" grade.

Composition per 1030 ml

Solution A	500.0 ml
Solution B	500.0 ml
Amino acid solution	20.0 ml
Vitamin solution	10.0 ml

pH 6.8 ± 0.2 at 25°C

Solution A:

Composition per litre:

$(NH_4)_2SO_4$	5.0 g
KH_2PO_4 (Unilab)	1.0 g
$MgSO_4.7H_20$	0.5 g
$CaCl_2.2H_2O$	0.1 g
NaCl	0.1 g
Wolfe's mineral solution	10.0 ml

Preparation of Solution A: Add components to distilled water and bring volume to 1.0 l. Mix thoroughly. Autoclave for 15 minutes at 15 psi pressure - 121°C. Cool to 45°- 50°C.

Wolfe's Mineral Solution:

Composition per Litre

$MgSO_4.7H_20$	3.0 g
Nitrilotriacetic acid	1.5 g
NaCl	1.0 g
$MnSO_4.H_2O$	0.5 g
$CaCl_2$	0.1g
$CoCl_2.6H_20$	0.1 g
$FeSO_4.7H_2O$	0.1 g
$ZnSO_4.7H_20$	0.1g
$AlK(SO_4)_2.12H_20$	0.01g

$CuSO_4.5H_2O$	0.01g
H_3BO_3	0.01g
$Na_2MoO_4.2H_2O$	0.01g

Preparation of Wolfe's Mineral Solution: Add nitrilotriacetic acid to 500.0 ml of distilled water. Dissolve by adjusting the pH to 6.5 with KOH. Add distilled water to 1.0 l. Add remaining components.

Solution B:

Composition per litre:

Agar (Gibco)	15.0 g
Cholesterol (Difco)	2.0 g
Tween 80 ® (Sigma)	1.0 g
Yeast extract (Gibco)	0.5 g

Preparation of Solution B: Add components to distilled water and bring volume to 1.0 l. Mix thoroughly. Gently heat to boiling. Autoclave for 15 minutes at 15 psi pressure-121 ° C. Cool to 45°-50 o C.

Amino Acid Solution:

Composition per 100 ml:

L-Histidine	0.5 g
DL-Methionine	0.1 g
DL-Tryptophan	0.1 g

Preparation of Amino Acid Solution: Add components to distilled water and bring to 100.0 ml. Filter sterilise.

Vitamin Solution:

Composition per litre:

myo-Inositol (Sigma)	200.0 mg
Calcium pantothenate (Sigma)	40.0 mg
Niacin (Sigma)	40.0 mg
Pyridoxine-HCL (Sigma)	40.0 mg
Thiamine (Sigma)	40.0 mg
p-Aminobenzoic acid (Sigma)	20.0 mg
Riboflavin (Sigma)	20.0 mg
Biotin (Sigma)	200.0 µg

Folic acid (Sigma) 200.0 μg

Preparation of Vitamin Solution: Add components to distilled water and bring to 1.0 l, filter sterilise.

Preparation of Medium: Combine 500 ml cooled sterile solution A and 500 ml cooled sterile solution B. Aseptically add 20 ml filter-sterilised amino acid and 10 ml vitamin solution. Adjust pH to 6.8. Pour into sterile petri dished or sterile tubes.

6.13 Tween 80 ® Medium (Patterson and Bridge, 1994)

Composition per litre:

Agar	15.0 g
Peptone	10.0 g
NaCl	5.0 g
$CaCl.2H_2O$	0.1 g
Bromocresol Purple (BDH, AnaLar)	0.025 g

Preparation of Medium: Add all components to distilled water and bring volume to 1.0l. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115°C . Pour into sterile petri dishes.

6.14 Tween ® 80 Medium II (Patterson and Bridge, 1994)

Composition per litre:

Plate count agar	23.5 g
$CaCl.2H_2O$	0.1 g
Tween 80 ®	1.0 ml

Preparation of Medium: Add all components to 1l distilled water. Mix thoroughly. Autoclave for 15 minutes at 15 psi pressure-121°C. Pour into sterile petri dishes.

6.15 Egg Yolk Agar (Smibert and Krieg, 1981)

Composition per litre:

Peptone	40.0 g
Agar	25.0 g
Na_2HPO_4	5.0 g
NaCl	2.0 g
Glucose	2.0 g
$MgSO_4$ 0.5% (wt/vol) solution	0.1 ml
Egg Yolk	1

Preparation of Egg Yolk: Surface sterilise the egg with 95% ethanol and leave to air dry. Aseptically separate the "egg yolk" from the "egg white" and add yolk to cooled sterile medium.

Preparation of medium: Add components, except egg yolk, to 1 l distilled water. Mix thoroughly. Autoclave at 15 psi pressure-121°C for 15 Minutes. Cool to 50°C Aseptically add the egg yolk to the cooled medium. Mix thoroughly to obtain an homogenous solution. Pour into sterile petri dishes.

6.16 Yeast Extract Agar (Atlas, 1993)

Composition per litre:

Agar	20.0 g
Tween 80®	10.0 ml
Glycerol	2.5 ml
Yeast extract	1.0 g
Buffer solution	2.0 ml

Buffer solution:

Composition per 400.0 ml

KH_2PO_4	60.0 g
Na_2HPO_4	40.0 g

Preparation of buffer solution: Add 40.0 g Na_2HPO_4 to 300.0 ml of distilled water. Mix thoroughly. Add 60.0 g KH_2PO_4. Mix thoroughly. Adjust pH to 6.0.

Preparation of medium: Add all components to distilled water and bring up to 1.0 l volume. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115°C. Pour into sterile petri dishes.

6.17 Yeast Extract Malt Extract Agar (Atlas, 1993)

Composition per litre:

Agar	20.0 g
Glucose	10.0 g
Tween 80®	10.0 ml
Glycerol	2.5 ml
Neopeptone	5.0 g
Malt extract	3.0 g
Yeast extract (Gibco)	3.0 g

Preparation of Medium: Add all components to distilled water and bring volume to 1.0 L. Mix thoroughly. Gently heat to boiling. Autoclave for 10 minutes at 15 psi pressure-115°C. Pour into sterile petri dishes.

6.18 Lipid Agar

Composition per litre:

Agar	20.0 g
Tween 80®	10.0 ml
Glycerol	2.5 ml

Preparation of Medium: Add all components to distilled water and bring to 1.0 L volume. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115°C . Pour into sterile petri dishes.

6.19 Bromocresol Purple Agar (Atlas, 1993)

Composition per litre:

Agar	20.0 g
Tween 80®	10.0 ml
Glycerol	2.5 ml
Bromocresol purple	0.04 g

Preparation of Medium: Add all components to distilled water and bring volume to 1.0 L. Mix thoroughly. Autoclave for 10 minutes at 15 psi pressure-115°C. Pour into sterile petri dishes.

6.20 Bromocresol Purple Milk Yeast Extract Agar (Atlas, 1993)

Composition per litre:

Milk solution	500 ml
Yeast extract solution	500 ml
Milk solution:	
Skim Milk Powder	40.0 g
Bromocresol Purple	0.08 g
Yeast extract solution:	
Yeast extract	20.0 g
Agar	15.0 g
Tween 80 ®	10.0 ml
Glycerol	2.5 ml

Preparation of Milk Solution: Add components to 500 ml distilled water. Mix thoroughly. Autoclave for 8 minutes at 11 psi pressure-116°C. Cool to 45°-50°C.

Preparation of Yeast Extract Solution: Add components to 500 ml distilled water. Mix thoroughly. Autoclave for 10 Minutes at 15 psi pressure-115^{0}C. Cool to 45°-50°C.

Preparation of Medium: Aseptically add cooled milk solution to cooled yeast extract solution. Gently mix together. Pour into sterile petri dishes and allow to dry.

6.21 Sweat Medium (Noble, 1981)

Composition per litre:

Agar	20.0 g
Arginine	0.00231 g
Aspartic acid	0.09065 g
Citrulline	0.00186 g
Glycine	0.03288 g
Glutamic acid	0.00417 g
Histidine	0.00834 g
Isoleucine	0.00369 g
Lysine	0.00510 g
Methionine	0.00093 g
Phenylalaine	0.00324 g
Proline	0.00510 g
Serine	0.04908 g
Taurine	0.00048 g
Valine	0.00603 g
Leucine	0.00510 g
Theonine	0.00834 g
Uric acid	0.00750 g
Urocanic acid	0.00570 g
Glucose	0.00300 g
Lactic acid	0.20000 ml
Urea	0.07400 g
Creatine	0.00046 g
NH_4OH	0.01000 ml
NaCl	0.14063 g
KCl	0.05795 g
$FeSO_4.7H_2O$	0.000596 g
KH_2PO_4	0.0002 g
$MgSO_4.7H_20$	0.02242 g
KI	0.00124 g

$MgCl_2.6H_2O$	0.00294 g
$MnCl_2.2H_2O$	0.00042 g
NaOH	0.06192 g
Acetic acid	0.00008 g
N-Methylnicotinamide	0.00480 g
4-Pyridoxic acid	0.00500 g
Folic acid	0.00090 g
Pantothenic acid	0.00010 g
Inositol	0.02100 g
p-Aminobenzoic acid	0.00020 g

Preparation of medium: Add all components except agar to 100 ml distilled water. Mix thoroughly. Filter sterilise. Add agar to 900 ml distilled water and autoclave for 15 minutes at 121° C. Allow to cool to 45°-50° C. Aseptically add the filter sterilised solution to the cooled agar. Mix thoroughly. Pour into sterile petri dishes.

6.22 Gelatin Agar

Composition per liter	
Gelatin (Gibco)	120.0 g
Nutrient Broth (Gibco)	8.0 g
Peptone	5.0 g
Meat extract	3.0 g
Tween 80 ®	10.0 ml
Glycerol	2.5 ml

Preparation of Medium:

Add all components to 1 l distilled water. Mix thoroughly and gently bring to boiling. Boil gently until all components are dissolved. Transfer to bijoux bottles and autoclave for 10 minutes at 15 psi pressure-115°C. Leave to set.

7. Appendix II

7.1 Phosphate Buffer Solution (PBS)

Composition per litre:

Na_2HPO_4 anhydrous	1.23 g
NaH_2PO_4	0.18 g
NaCl	8.50 g

Preparation of solution: Add all components to 1.0 l distilled water. Mix thoroughly. Autoclave for 20 minutes at 15 psi pressure-121°C.

7.2 Phosphate Sorbitol Buffer Solution (PSBS)

Composition per litre:

Sorbitol	93.0 g
Na_2HPO_4 anhydrous	1.23 g
NaH_2PO_4	0.18 g
NaCl	8.50 g

Preparation of solution: Add all components to 1.0 l distilled water. Mix thoroughly. Autoclave for 20 minutes at 15 psi pressure-121°C.

7.3 Amphotericin B Solution

Amphotericin B (Sigma)	51 mg
Sodium deoxycholic acid (Sigma)	49 mg
PBS buffer	10.0 ml

Preparation of Amphotericin B Solution: Add components to PBS. Mix thoroughly. Filter sterilise.

7.4 Nystatin Solution

Nystatin (5060 U/mg, Sigma)	1.0g
Ethanol (95%)	10.0 ml

Preparation of Nystatin Solution: Add components together. Mix thoroughly. Filter sterilise.

7.5 ID 32 C Identification System

Components of System (as specified in bioMérieux Identification System manual)

Suspension Medium

Distilled water	2 ml

C Medium

Ammonium sulphate	5.0 g
Monopotasium phosphate	0.31 g
Dipotasium phosphate	0.45 g
Disodium phosphate	0.92 g
Sodium chloride	0.1 g
Calcium chloride	0.05 g
Magnesium sulphate	0.2 g
Histidine	0.005 g
Tryptophan	0.02 g
Methionine	0.02 g
Agar	0.5 g
Vitamin solution	1 ml
Trace elements	10 ml
Demineralized (distilled) water	1000 ml

ID 32C Identification Trays Contain:

Sorbitol	Galactose
D-xylose	actidione (cycloheximide)
Ribose	sucrose (saccharose)
Glycerol	N-acetyl-glucosamine
rhamnose	DL-lactate
palatinose	L-arabinose
erythritol	cellobiose
melibiose	Raffinose
glucuronate	Maltose
Melezitose	Trehalose
Gluconate	2-keto-gluconate
Levulinate	α-methyl-D-glucoside
Glucose	Mannitol
Sorbose	Lactose
Glucosamine	inositol
Esculin	Control

Inoculate 2 ml sterile distilled water with test organism to a turbidity of McFarlands Scale 2. Transfer 250 µl aliquot's of this to C Medium. Mix Well. Transfer 135 µl aliquot's of this to each well of identification strip. Incubate in a moist container for at 37 °C for 48 hours. Results are scored on ID 32C Identification strips and analysed by Nicola Egerton, Medbio Enterprises Limited.

7.6 Azelaic Acid Solution

Composition per 10 ml:

Azelaic Acid (Sigma®)	2 g
Distilled water	10 ml

Preparation of Solution:

Add azelaic acid to distilled water and gently heat to dissolve. Cool to 37°C and use quickly as acid comes out of solution on cooling.